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Departamento de Ciências Médicas

Ana Luísa Ferreira Graça

Predictors of aging and potential of FTIR to detect protein aggregates

Preditores do envelhecimento e deteção de agregação proteica por FTIR

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Carla Alexandra Pina da Cruz Nunes, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro.

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Dedico este trabalho aos meus pais e à minha irmã, uma vez que são as pessoas mais importantes da minha vida.

"O importante é não parar nunca de questionar"
Albert Einstein

o júri

presidente

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palavras-chave

Envelhecimento, Características do Envelhecimento, Biomarcadores do Envelhecimento, Doenças Relacionadas com o Envelhecimento, Metabolómica, FTIR.

resumo

O envelhecimento é definido como um processo fisiológico complexo acompanhado de um declínio progressivo das estruturas biológica, psicológica e social dos indivíduos. A análise de metabolitos é cada vez mais utilizada em diversos estudos da população humana, com a finalidade de compreender os processos fisiológico e patológico do indivíduo. Sabe-se que o crescente envelhecimento da população é a origem de enormes desafios nos cuidados de saúde e no tratamento de doenças relacionadas com a idade, sendo o "envelhecimento" um campo de pesquisa promissória. O metaboloma humano varia ao longo da vida e essas alterações estão possivelmente relacionadas com o aparecimento de várias doenças associadas à idade, sendo expectável que o estudo do metaboloma do envelhecimento possa contribuir para a compreensão da sua fisiologia molecular e das doenças relacionadas com este. A utilização de técnicas de análise metabolómica, como o FTIR, pode ajudar a identificar quais os preditores biológicos do envelhecimento, contribuindo para a compreensão dos mecanismos envolvidos no seu processo. O FTIR é uma técnica que tem recebido especial atenção no campo clínico, uma vez que utiliza as frequências de vibração das ligações químicas das moléculas presentes nas várias amostras analisadas para a obtenção de uma “impressão digital” metabólica específica para cada amostra de forma rápida e barata.

Esta dissertação tem como objetivo identificar as diferenças ao nível das proteínas e agregados proteicos presentes em diferentes amostras através da aplicação do FTIR na região do infravermelho médio.

Numa primeira abordagem, a técnica de FTIR foi aplicada a amostras de leveduras de forma a identificar alterações nas proteínas. Foram ainda aplicadas metodologias de análise multivariada (PCA e PLS) com o objetivo de distinguir, ao nível das proteínas, amostras de leveduras wild-type e stress (com indução de agregados). Como a técnica de FTIR demostrou ser capaz de identificar alterações proteicas em amostras de levedura foram usadas amostras de plasma humano de indivíduos compreendidos entre os 65 e 82 anos de idade. Recorrendo à mesma metodologia estudaram-se amostras de plasma de indivíduos com diferentes idades, pretendendo-se relacionar a idade com a presença de agregados proteicos/alteração da conformação proteica.

Os espectros compreendem um vasto conjunto de comprimentos de onda, no entanto, apenas a região 1800-1350 cm^{-1} foi alvo de um maior estudo, visto que está maioritariamente relacionada com a conformação das proteínas e com a presença de agregados proteicos.

A técnica de FTIR tornou-se um método promissor de screening, uma vez que demonstrou ser uma técnica capaz de contribuir para a compreensão do processo de envelhecimento.

keywords

Aging, Hallmarks of Aging, Aging Biomarkers, Age-Related Diseases, Metabolomics, FTIR.

abstract

Aging is defined as a complex physiological process accompanied by a progressive decline of biological, psychological and social structures of individuals. Metabolite analysis is being used in several studies of human populations in order to understand physiological and pathologic processes of an individual. It is known that the increasing aging of population is the origin of enormous challenges to healthcare provision and treatment of age-related diseases, being "aging" a promissory research field. Human metabolome varies throughout life and those changes are certainly related to the onset of several age-related diseases. It is expectable that the study of aging metabolome could help to understand molecular physiology of aging and age-related diseases. The use of metabolic analysis techniques, such as FTIR, may help to identify the biological predictors of aging, which allow understanding the mechanisms involved in the aging process. FTIR technique has received special attention in the clinical field, once it uses the vibration frequencies of the chemical bonds of molecules present in the analyzed sample to yield a metabolic fingerprint specific for each molecule quickly and cheaply.

This dissertation pretends to identify the differences at the protein level and protein aggregates in different samples, through the application of FTIR spectroscopy in the medium infrared region.

In a first approach, the FTIR technique was applied to yeast samples in order to identify protein alterations. Multivariate data analysis methodologies (PCA and PLS) were also applied in order to distinguish, at the protein level, wild-type and stress (with aggregate induction) yeast samples. FTIR technique has been shown to be capable of identifying protein changes in yeast samples, so human plasma samples from individuals between 65 and 82 years old were used. Using the same methodology, plasma samples from individuals of different ages were studied, with the goal to relate age with the presence of protein aggregates/alteration of protein conformation.

The spectra comprise a wide set of wavelengths, however, only the 1800-1350 cm^{-1} region of the spectrum received larger study, once it is mostly related with protein conformation and with the presence of protein aggregates.

The FTIR technique has become a promising screening method, since it has shown to be a technique capable of contributing to the comprehension of the aging process.

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Abbreviations

A	Absorbance
Aβ	Amyloid β
Aβ42	Amyloid β42
AB	Aging Biomarkers
AD	Alzheimer's Disease
ANNS	Artificial Neural Networks
APP	Amyloid-Precursor Protein
ARD	Aging-Related Diseases
ATR	Attenuated Total Reflectance Spectroscopy
BMI	Body Mass Index
CA	Cluster Analysis
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CHF	Congestive Heart Failure
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone Sulfate
DNA	Deoxyribonucleic Acid
E_2	Estradiol
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionization
ESR	Electron Spin Resonance Spectroscopy
FEV1	Forced Expiratory Volume In 1 Second
FIR	Far-Infrared
FISH	Fluorescence *In Situ* Hybridization
F_2-IsoP	F2-Isoprostanes
FSH	Follicle-Stimulating Hormone
FTIR	Fourier Transform Infrared Spectroscopy
FT	Fourier Transform
GC	Gas Chromatography
GC-MS	Gas Chromatography–Mass Spectrometry
GC/NICIMS	Gas Chromatography/Negative Ion Chemical Ionization Mass Spectrometry
GC-TOF-MS	Gas Chromatography-Time-of-Flight-Mass Spectrometry
GSH	Glutathione

HbA1c	Glycosylated Hemoglobin
HDL	High Density Lipoprotein
H3K9me3	Trimethylated Lysine at the 9 Position of Histone H3
H3K27me3	Trimethylated Lysine at the 27 Position of Histone H3
HPA-axis	Hypothalamic–Pituitary–Adrenal Axis
HPLC	High-Performance Liquid Chromatography
HSP	Heat Shock Proteins
IGF-1	Free Insulin-Like Growth Factor-1
8-iso-$PGF_{2\alpha}$	8-Isoprostaglandin $F_{2\alpha}$
IL-6	Interleukin-6
IR	Infrared
LC	Liquid Chromatography
LC-MS	Liquid Chromatography–Mass Spectrometry
LDA	Linear Discriminant Analysis
LDL	Low Density Lipoprotein
LH	Luteinizing Hormone
MI	Myocardial Infarct
MIR	Mid-Infrared
mRNA	Messenger RNA
MRS	Magnetic Resonance Spectroscopy
MS	Mass Spectrometry
MSI	Metabolomics Standard Initiative
mtDNA	Mitochondrial DNA
N	Noise
NIR	Near-Infrared
NMR	Nuclear Magnetic Resonance
8-OHdG	8-Hydroxy-2-Deoxyguanosine
PCA	Principal Component Analysis
PCR	Principal Components Regression
PCs	Principal Components
PEF	Peak Expiratory Flow
PG	Prostaglandins
PLS	Partial Least Squares
PLS-DA	Partial Least Squares Discriminant Analysis
p-Tau	Phosphorylated Tau
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
R^2	Determination Coefficient
RIA	Radioimmunoassay
RMSEC	Root Mean Square of Calibration
RMSEP	Root Mean Square Error of Prediction
ROS	Reactive Oxidative Species
RT-PCR	Real Time Polymerase Chain Reaction

S	Signal
SA-β-gal	Senescence-Associated β-Galactosidase
SAHF	Aging-Associated Heterochromatin Foci
SBP	Systolic Blood Pressure
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SNR	Signal-to-Noise Ratio
SNS	Sympathetic Nervous System
SOD	Superoxide Dismutase
T	Transmittance
TEM	Transmission Electron Microscopy
tHcy	Total Homocysteine
TNF-α	Tumor Necrosis Factor-α
t-Tau	Total Tau
UV	Ultraviolet
VLDL	Very Low Density Lipoprotein
VO_{2max}	Maximum Volume of Oxygen
WBC	White Blood Cell Count

Chapter One

This chapter defines and characterizes aging and summarizes its main theories and senescence mechanisms. Also, clarifies the importance of aging biomarkers, describes the hallmarks of aging and identified biological predictors of aging. An introduction of the Fourier Transform Infrared spectroscopy technique as novel diagnostic modality of aging is also made. The aims of the work are presented in the final part of the chapter.

1. INTRODUCTION

Aging is defined as a complex physiological process characterized by a persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration [1]. It is mainly caused by genetic and environmental influences such as diet, physical exercise, exposure to pollutants and microorganisms: these factors explain how two people with the same chronological age differ widely in their physical appearance and physiological state [2]. Some of the consequences of aging process are loss of mobility and agility, weakness, end of the reproductive capacity and debility of the immune system – immunosenescence – which implies higher vulnerability for cancer, autoimmune diseases or infections and neurodegeneration [3–5]. Instead of prolonging lifespan at all costs it is vital to have an increased healthspan, the portion of lifespan throughout which function is satisfactory to preserve autonomy, productivity and well-being. Due to the increasing aging population, the healthcare has to surpass several challenges [6]. Therefore aging biomarkers (AB) are an urgent need to measure the health state of elderly and assess the effect of novel treatments and it is essential too, once it will be beneficial to determinate the biological age [7].

This work aims to systematically review the biological predictors of aging that can be useful to interpret and extract the information obtained by metabolomic assays. The potential of FTIR as a metabolic tool to contribute to aging metabolic fingerprint is also discussed.

2. THEORIES OF AGING

The enigmatic nature of aging makes its study one of the greatest challenges of science. Despite advances in biological knowledge and molecular approaches to analyze biological samples, the exact answer to the biggest questions: why and how organisms age are still unknown. Over the decades many theories have emerged to describe aging. For instance, Medvedev catalogued about 300 and the number is still growing [8,9].

2.1. Evolutionary Theories of Aging

The oldest arguments about the evolution of aging were written in 1858 by Darwin & Wallace and were related with natural selection [10]. However it was August Weismann who debated for the first time the problem about how aging evolved [11] and created the first evolutionary theory of senescence - the theory of programmed death - based on natural selection [12]. This theory proposes that aging leads to the improvement of the species and not the individual. According to this theory, there is an exact mechanism of death projected by natural selection to remove older

individuals therefore they would no longer compete with the younger generations. The biological mechanism of this theory is explained by the existence of a precise limitation on the number of divisions in somatic cells [12,13]. This speculation was confirmed by Hayflick and Moorhead in 1961 [14], when they found that fibroblasts have a limited number of cell divisions. However, Weismann abandoned this theory later, considering that organisms should be able to apply further resources to reproduce [13]. Based on this idea, Thomas Kirkwood created the disposable soma theory suggesting that the somatic cells are preserved only to guarantee the success of reproduction process, becoming disposable after this period [15], and foresees that aging arises due to the increasing of cellular damages [16].

Later, Peter Medawar, a British professor of zoology and anatomy, proposed the mutation accumulation theory of aging. He hypothesized that if a deleterious mutation was limited to long-lived organisms, this will be passed to the next generation before the prejudicial effect of it becomes evident [13,17,18]. Finally, the antagonistic pleiotropy theory of aging was created by George C. Williams and he suggested that senescence and cellular damage are the consequence of pleiotropic genes or genes with several phenotypic effects and that natural selection selects genes that increase the probabilities of success in early life regardless of those genes have harmful effects in later life [19,20].

2.2. Senescent Theories of Aging

The evolutionary theories of aging explain why we age but the mechanisms of senescence give us the answer to how we age and how aging disturbs homeostasis. These mechanisms are generally controversial and some of them have more credibility than others but all have scientific validation [8,21].

In 1956 Harman proposed the free radical theory [22], that considers that aging is a result of the ineffectiveness of the antioxidant systems. About 90% of reactive oxygen species (ROS) (O_2, O_2^- and OH) are produced in mitochondria through oxidative phosphorylation [23]. In 1963 Leslie Orgel [24] suggested the error catastrophe theory, describing that aging causes a decline in fidelity of genetic expression leading to errors in protein synthesis. Errors in mRNA and/or proteins would compromise the balance of the cell. Most of these accumulated genes are eliminated by repair systems, but occasionally it does not function properly, causing an accumulation of genomic errors [25]. Two years later, in 1965, Hayflick created the telomere theory [26]. Telomeres are repetitive DNA sequences located at the end of each human chromosome. In every cell division, telomeres become increasingly shorter, once replicative DNA

polymerases are not capable of replicating telomeres and telomerase is typically expressed in germ cells and only in some adult stem cells. Sooner or later the cell finishes growing and gets into senescence [25,27]. Later, in 1970, Kanungo proposed the gene regulation theory (reviewed in [28]). It defines that differentiation, growth, maturity and senescence have their own regulation mechanisms. The products/sub-products of each phase can trigger the following phase by activating or repressing specific genes. So, senescence is the consequence from alterations in gene expression with aging [28]. In 1972, Harman described the function of free radicals in mitochondrial damage creating the mitochondrial theory [22,29]. Mutations in mitochondrial DNA (mtDNA) lead to a decrease on oxidative phosphorylation, decreasing the production of energy and consequently increasing ROS [25,29]. Fabris, one of the researchers studying the relation between immune, neurological and endocrine systems and aging created the immune-neuro-endocrine theory in 1990, which proposes that the loss of plasticity between immune and neuroendocrine systems leads to aging. This theory is based on the existence of a functional decline of immune system and a growth of autoimmune factors [30,31]. Finally, in 2009, Chung *et al.*, related inflammation and aging, creating the inflammation theory [32]. During aging there is a dysregulation of the immune system and a modification of the redox status. The inflammatory mechanism is associated to aging and age-related diseases (ARD) and it works like a linkage among normal and pathological aging. The upregulation of pro-inflammatory intermediaries which are activated by aging due a redox imbalance leads to activation of different pro-inflammatory signaling pathways [32].

3. THE HALLMARKS OF AGING

Aging is caused largely by accumulation of cellular damage. Some pathologies associated with aging, like inflammation and atherosclerosis, imply uncontrolled cellular growth or hyperactivity [33]. Based on these, a few critical questions have amounted about the physiological bases of aging. López-Otín *et al.*, 2013 identified and characterized nine cellular and molecular hallmarks of aging which contribute to the aging process and that all together define the aging phenotype [34].

Each one of the nine hallmarks must satisfy the ensuing criteria: (1) should be evident in normal aging; (2) experimental aggravation must hasten aging and (3) experimental improvement should delay the normal aging process and consequently increase healthy lifespan. These nine hallmarks of aging are distributed in three categories: primary hallmarks, antagonistic hallmarks and integrative hallmarks [34] (figure 1).

The primary hallmarks have in common the fact that are all clearly harmful. One of the primary hallmarks of aging is telomere attrition. The progression of cell divisions takes to attrition of chromosomes ends [9,35,36]. When the telomeres reach a certain short length, cell senescence is triggered, known as replicative senescence, linked to telomere attrition [36,37]. During aging there is also a lot of genetic and epigenetic alterations that are responsible to regulate lifespan, as well as a decrease in protein homeostasis, which is responsible for some age-related diseases [38]. The consequences from extrinsic and intrinsic DNA damage include translocations, point mutations, chromosomal losses and gains and telomere shortening. It is also known that defects in nuclear architecture lead to genome instability and result in premature aging syndromes [34,39–42].

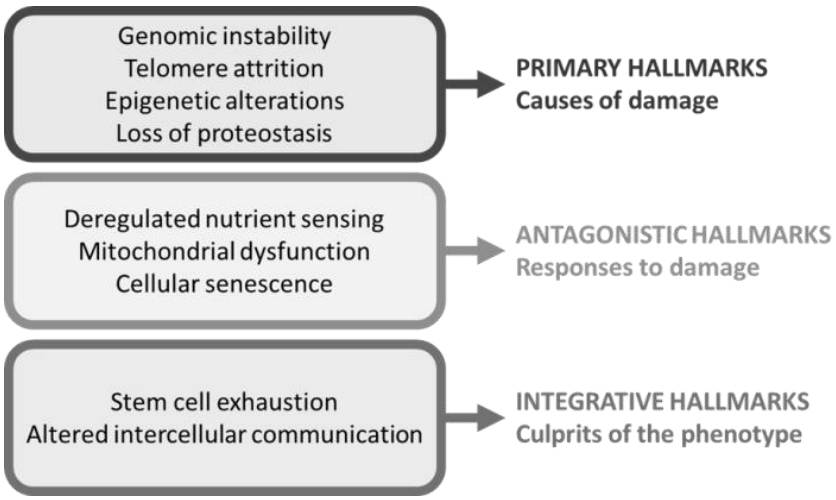

Figure 1 - The nine suggested hallmarks of aging grouped into three categories: primary hallmarks, responsible for cellular damage; antagonistic hallmarks, which are part of compensatory or antagonistic answers to the damage and integrative hallmarks, which firstly mitigate the damage, but become deleterious if aggravated. The relation between these hallmarks characterize to the functional decline related with aging. Adapted from [34].

Antagonistic hallmarks have contradictory effects based on their intensity once at low levels they lead to beneficial effects. In this class of hallmarks it is possible to consider mitochondrial dysfunction, deregulated nutrient sensing and cellular senescence. Aging is related with the accumulation of somatic mitochondrial DNA mutations and also the aging process is characterized by severe metabolically alterations in the body. In the period of food plenty, nutrient sensing pathways involve anabolism and storage, and scarcity activates homeostatic mechanisms, such the mobilization of internal stores through autophagy for example [43]. Nutrient and stress sensors intervene in extend lifespan that happen in response to various physiological and environmental signals such as dietary restriction which is responsible for a rapid decrease in the

mortality rate, neutralizing the causes of aging in an acute way [44]. During aging there is an irreversible arrest of cell proliferation, known as cellular senescence [34]. This antagonistic hallmark of aging can be an advantageous compensatory response to the damages which become deleterious and accelerate aging when tissues deplete their regenerative capability [34].

The third category, integrative hallmarks, comprises stem cell exhaustion and altered intercellular communication which affect homeostasis [34]. These hallmarks are all interrelated, however there are still many challenges in comprehending the aging process [34,45]. Adult stem cells are indispensable for normal tissue homeostasis and repair [46]. The exhaustion of adult stem cell pools are the result, for example, of premature senescence and deranged metabolic signaling and there is a strong evidence that altered and decreased function of adult stem cells has a significant role in the beginning of the aging diseases [47]. Aging also contributes to alterations in intercellular communications and the most relevant age-related alteration in intercellular communication is “inflammaging”, a smoldering pro-inflammatory phenotype that accompanies aging in mammals, involved in age-related diseases [34,48].

4. PHYSIOLOGICAL AND PREMATURE AGING

Aging is an inevitable stage of the development and can be classified in two different types: the physiological aging (senescence) which refers to intrinsic, progressive and universal changes related to advancing age, and the pathological aging (senility) related to modifications which take place due to non-normative factors such diseases, brain trauma or poor life habits, independent of modifications correlated with healthy aging [49].

Physiological changes are common to everyone, however their beginning and intensity are different. These changes are mainly related with a decrease in metabolic rate, reduction in cell counts, reduction of body fluids and tissue atrophy [25]. Some researchers compile these alterations into three distinct groups: the first group covers the cellular homeostatic mechanisms such as corporal temperature and extracellular fluid volumes, the second refers to the decrease in the mass of organs and finally the third is correlated with the decline of the functional reserves of the body [2]. As a consequence, all these physiological changes may lead to several abnormalities in neurologic, cardiopulmonary, immunologic, endocrine and motor functions. Thus, the contact with risk factors as alcohol, smoking, obesity, hypertension, dyslipidemia and stress may lead to a wide range of diseases such as neurodegenerative diseases, heart failure, atherosclerosis, arrhythmias, osteoporosis, diabetes, infections and tumors [25].

The pathological and non-pathological aging is caused by physiological and environment

changes, which often compromise life quality. However, aging may also be triggered by genetic mutations which lead to some known diseases such as Progeria, Werner, Bloom, Cockayne and Rothmund-Thomson syndromes and Fanconi anemia, which can be an ideal model for understanding age-related genetic alterations [42]. These patients develop characteristics of accelerated aging due to mutations in a couple of genes implied in genetic stability. Clinically these syndromes mostly involve premature senescence (huge risk of cancer, atherosclerosis, gray hair), metabolic disorders (hyperlipidemia, diabetes), skin changes (hyperkeratosis, atrophy, ulcer) and senile dementia [42].

5. BIOLOGICAL PREDICTORS OF AGING

The search of specific biological predictors of aging is a challenge for scientists. The main goals of identifying biomarkers of aging are related to the comprehension of the mechanisms involved in the process, determinate the rate of aging in an individual, observing the impact of interventions, like exercise or food restriction in the aging process and estimate lifespan by determinate the biological age [50].

A biomarker can be defined as a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or pharmacological response to a therapeutic intervention [39]. Molecular biomarkers can be classified into three categories: those that control the development of the diseases, those that identify the effect of the medicines and those which are defined as surrogate endpoints in clinical trials [39]. A surrogate marker serves to monitor and diagnose the progression of the diseases [51] and the surrogate endpoints predict the effects of therapeutic interventions and are a substitute of clinical endpoints [39].

The term “aging biomarker” appeared for the first time in gerontology and geriatry in the early 1980s [52]. An aging biomarker is described as a biological parameter that will envisage the functional competence in advanced age [50]. Baker & Sprott refer an AB as a biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, predict the functional capacity at some later age [53,54]. Many researchers tend to assume that an age-related change in a biological factor is considered an AB. Nonetheless, for a biological parameter be considered an AB it is necessary to distinguish it from a modification indirectly allied with age. Differentiate age biomarkers of disease biomarkers is not an easy process, so there are some specific criteria for it, which are different depending on the scientist who wrote them [50,52–55].

Many factors are directly affected and changed over aging process and a few markers are being used to predict biological aging [56]. These predictors might be used to identify people at huge risk of developing an age-associated disease or disability. Determination of biological age has acquired significance in several subjects such cardiology, neurology or transplant medicine, as it can be a powerful indicator to estimate the quality of tissues and organs [54].

Various putative AB have appeared in the past few decades. Table 1 shows some biological predictors of aging identified until now, which are regularly used as AB in population-based studies and applied in a variety of settings capable of differentiating amongst healthy and unhealthy aging in elderly [7,57]. As it is possible to see in table 1, there are several parameters able to characterize and quantify vital functions prone to decline during aging. The molecular predictors of aging are associated to the domains of physiological, endocrine, immune and inflammatory functions, nervous system and molecular mechanisms [57,58]. The majority of these parameters are frequently part of routine medical examinations and for this reason they are reasonably easy to measure [59].

The molecular changes which disturb the function of organs, tissues and cells related to physiological function are a remarkable group of biomarkers candidates. To assess lung function it is important to measure FEV1 (forced expiratory volume in 1 second) [60], VO_{2max} (maximum volume of oxygen) [61] and PEF (peak expiratory flow) [62], which are three important biomarkers of pulmonary health. In cardiovascular function the biomarkers that allow to assess the risk of coronary heart disease are systolic blood pressure (SBP) [63], diastolic blood pressure (DBP) [64], pulse pressure [65], resting pulse rate [66], total homocysteine (tHcy) [67] and electrocardiogram [68]. To evaluate bone health and body composition it is essential estimate the bone mass, bone density [69], muscle mass [70] and body mass index (BMI) [71]. To assess glucose metabolism it is important to quantify the fasting glucose [72] and the glycosylated hemoglobin (HbA1c) [73]. On the other hand to assess other metabolic processes it is crucial to assess the following biomarkers: low density lipoprotein (LDL) [74], very low density lipoprotein (VLDL) [74], leptin [75] and adiponectin [76].

Biological predictors of aging related to endocrine function are grouped in three different categories: HPA-axis (hypothalamic–pituitary–adrenal axis) markers, which are dehydroepiandrosterone sulfate (DHEA-S), dehydroepiandrosterone (DHEA) [77,78], cortisol [79] and DHEA-S:cortisol ratio [80]; markers of sex hormones, which are E_2 (estradiol), FSH (follicle-stimulating hormone), LH (luteinizing hormone) [81] and testosterone [82] and the marker of growth hormones which is IGF-1 (free insulin-like growth factor-1) [83]. These markers are all

associated to higher risk of premature mortality and physical frailty.

The changes in immune system in elderly are associated with alterations in B cells [84], T cells [85] and white blood cell count (WBC) [86]. In what concerns inflammatory response, age-related modifications are mainly associated with changes in the levels of C-reactive protein (CRP) [87], interleukin-6 (IL-6) [88], tumor necrosis factor-α (TNF-α) [89], fibrinogen [90] and albumin [91], which are considered AB.

In nervous system biological predictors of aging are distributed in two different groups: the markers of central nervous system (CNS), which are amyloid β42 (Aβ42) [92], total Tau (t-Tau) [93,94], phosphorylated Tau (pTau) [95] and F_2-isoprostanes [96], linked also to Alzheimer's disease, and the markers of sympathetic nervous system (SNS) which are norepinephrine (noradrenaline) [97] and epinephrine (adrenaline) [98].

The domain with more identified AB to date is the molecular mechanisms domain. According to the senescent theories of aging there are a lot of molecular mechanisms of aging. Relatively to oxidative stress, the accepted markers are reactive oxidative species (ROS) [99], superoxide dismutase (SOD) [100], 8-hydroxy-2-deoxyguanosine [101], 8-isoprostane [102], glutathione [103], serum Hsp70 [104] and tyrosine [105]. Concerning genomic instability mechanisms, the accepted biomarkers are chromosome structural aberrations levels [106], DNA repair activity [107,108], $p16^{INK4A}$ expression level [109], p21 expression level [110], p53 expression level [111], p53-binding protein 1 level [112] and number of deletions in mitochondrial DNA [113]. The biomarkers of senescence are the levels of senescence-associated β-galactosidase [114], apoptosis levels [115], number of senescent cells [116] and Ink4a/Arf ratio [117]. Concerning epigenetic alterations, the number of γ-H2AX foci [118], trimethylated lysines at the 27 and 9 positions of histone H3 [119,120] and senescence-associated heterochromatin foci [121] are considered AB. Relatively to telomere attrition the established AB are telomere length [122] and telomerase activity [123]. Finally shape of nuclear lamina [124], progerin level [125] and wound healing rate [126,127] are other markers related with the molecular mechanisms of aging (table 1).

5.1. Stages of AB Development

The identification of new biomarkers is required to improve the diagnosis, guide the target molecular therapy and observe the therapeutic response in an extensive range of diseases. For instance, proteomic methods based on mass spectrometry (MS) ensure the development of new biomarkers which potentiates the development of new blood tests. Therefore, advances in technology enabled the conception of a biomarker pipeline usually assessed in a series of

preclinical and clinical phases [128,129] (figure 2). Plasma is a good candidate for the development of new biomarkers as it may reflects the pathological and physiological processes that are underway in the entire body [129,130]. In proteomic-based methods the targets for biomarker discovery are thousands of proteins/peptides that are typically quantified and compared between normal and illness state. Nevertheless, biomarker detection is not actually the issue, the problem is in the verification and validation of these putative biomarkers [128–131]. Another way to select an aging biomarker is establish a correlation among putative biomarkers and the chronological age and telomere restriction fragments [128,132,133]. The next step in biomarker discovery is verification, which is essential to recognize biomarkers to proceed to clinical validation. In this phase, putative biomarkers are evaluated for changes triggered by biological, environmental and/or genetic factors [133,134]. The last step is validation, where the samples are examined for biomarker specificity and sensitivity, as well as its standardization potential, before further clinical evaluation and use in clinical routine. This phase is one of the hardest steps in AB development because it is hard to predict if a molecule is really an indicator of the aging status. However, there are also some factors that limit healthspan that difficult biomarker validation, such as frailty, which is defined as an age-related clinical syndrome that entails loss of resilience and failure to recover from acute problems (e.g. pneumonia, stroke and fractures). Therefore there are four important requirements in the validation of an AB candidate: (i) evidence better predictive power than chronological age, (ii) give information about the age range, (iii) establish specificity and sensitivity and (iv) analyze and minimize methodological discrepancies amongst laboratories [133–135].

Table 1 - Briefly summary of the main biological predictors of aging.

Biological Predictors of Aging					
Subdomain	**Biomarker**	**Health indication**	**Related pathologies**	**How to measure**	**References**
Domain: Biomarkers of Physiological Function					
Lung Function	Forced expiratory volume in 1 second (FEV1)	From age 25 years, FEV1 declines around 25ml/year in woman and 32 ml/year in men. Several studies documented that there is an inverse association among mortality and FEV1.	Mortality, CVD, Pulmonary diseases	Spirometry exam Measures FEV1.	[60]
	VO_{2max} (maximum volume of oxygen)	10% of VO_{2max} declines *per* decade in woman and men.	Mortality, CVD, Pulmonary diseases	Spirometry exam VO_{2max} measures the functional limit of cardiovascular system being described as index of cardiorespiratory fitness.	[61]
	Peak expiratory flow (PEF)	Indicator of airway limitation.	Asthma, COPD	Spirometry exam Measures the airway obstruction.	[62]
Cardiovascular Function	Systolic blood pressure (SBP)	The highest arterial blood pressure in an artery which occurs when the heart is pumping blood for body.	Stroke, CHD, Mortality	Physical exam Measures heart activity.	[63]
	Diastolic blood pressure (DBP)	The lowest arterial blood pressure which occurs when the heart is resting.	Stroke, CHD, Mortality	Physical exam Measures heart activity.	[64]
	Pulse pressure	Indicator of augmented arterial stiffness.	Stroke, CHD, MI, Mortality	Physical exam Measures heart activity.	[65]
	Resting pulse rate	Indicator of heart functioning and measure of overall fitness.	CHD, Mortality	Physical exam Measures heart activity.	[66]
	Total homocysteine (tHcy)	tHcy is an amino acid involved in lipid metabolism. Higher plasma homocysteine levels might cause toxicity by a diversity of mechanisms, such as oxidative damage, which has been related to an enhanced rate of aging.	Cardiovascular, Peripheral vascular, Cerebrovascular diseases	Non-fasting plasma using reverse-phase HPLC with isocratic elution	[67]
	Electrocardiogram	Indicator of cardiac function.	Stroke, Cardiovascular risk, Mortality	Physical exam Measures the electrical impulses in the heart.	[68]
Bone Health	Bone mass	Bone mass and density decrease in men and women with age.	Mortality, Fractures, CVD	Bone densitometry exam	[69]
	Bone density				
Body Composition	Muscle mass	Aging is associated with reduced muscle mass,	Mortality	Physical exam Assesses muscle	[70]

		especially leg muscle mass.		mass.	
	Body mass index (BMI)	Indicator of the equilibrium among energy intake and expenditure besides, it is also an indicator of malnutrition. BMI is positively related with prevalent disability.	Mortality, CVD, Diabetes mellitus, Stroke	Physical exam Measures the body fat based on height and weight.	[71]
Glucose Metabolism	Fasting glucose	Monitor the quantity of sugar in blood.	Diabetes, CHD, Mortality	Hexokinase Method	[72]
	Glycosylated hemoglobin (HbA1c)	Monitor the quantity of sugar linked to hemoglobin in erythrocytes.	Diabetes	HPLC Assay Fasting blood (plasma)	[73]
	Low density lipoprotein (LDL)	Makes the transportation of cholesterol from the liver to be integrated in cell membrane tissues.	Stroke, CHD, Atherosclerosis	ELISA using immunopurified polyclonal antibodies Fasting blood (plasma)	[74]
	Very low density lipoprotein (VLDL)	Makes the transportation of cholesterol, phospholipids, endogenous triglycerides and cholesteryl esters.	Atherosclerosis, Coronary artery disease	ELISA using immunopurified polyclonal antibodies. Fasting blood (plasma)	[74]
	Leptin	Is a protein hormone, which regulates energy expenditure and food intake. There is a progress of central resistance to leptin with aging.	Diabetes mellitus, Metabolic syndrome (dyslipidemia, hyperglycemia, hypertension, abdominal obesity), Atherosclerosis, Osteoporosis	Leptin concentrations were measured with a commercial RIA kit	[75]
	Adiponectin	Anti-inflammatory protein secreted by adipocytes and has a protective function against insulin resistance and atherosclerosis. Adiponectin increases with aging and there is a strong association among this protein and mortality.	Metabolic syndrome (dyslipidemia, hyperglycemia, hypertension), MI	ELISA Fasting blood (plasma)	[76]
Domain: Biomarkers of Endocrine Function					
HPA-axis	Dehydroepiandrosterone sulfate (DHEA-S)	DHEA is a cortisol antagonist and a steroid hormone produced from cholesterol. DHEA-S is a metabolite of DHEA. The levels of these adrenal steroids decline with age.	AD, Mortality	RIA	[77,78]
	Dehydroepiandrosterone (DHEA)				
	Cortisol	Steroid hormone, produced by the adrenals, which implies in body answer to physiological stress.	CVD, Fractures, Poor cognitive function, Mortality	RIA	[79]
	DHEA-S:Cortisol ratio	The DHEA-S:Cortisol ratio is related with	Mortality	DHEA-S and Cortisol	[80]

		cognitive impairment.		determined through Coat-A-Count kit	
Sex-Hormones	Estradiol (E_2) FSH (Follicle-stimulating hormone) LH (Luteinizing hormone)	Responsible for secondary sexual characteristics in women.	Mortality	RIA	[81]
	Testosterone	Steroid hormone. Men senescence is characterized by a reduction in plasma testosterone levels	Mortality	GC (plasma)	[82]
Growth Hormone	Free insulin-like growth factor-1 (IGF-1)	Growth factor, inhibitor of apoptosis and controls cells growth and development.	CAD, Mortality, Osteoarthritis	RIA	[83]
Domain: Biomarkers of Immunity and Inflammation					
Immune Function	B cells	Aging is characterized by lack of B lymphocytes. In elderly it is normal a decrease of B cell number and diversity linked with poor health status.	Lymphoid neoplasms (chronic lymphocytic, leukemia, multiple myeloma)	Flow Cytometry	[84]
	T cells	T lymphocytes protect the organism against pathogens. CD4:CD8 ratio increases very slowly with age and this ratio less than 1 leads to high mortality.	Mortality, AD, Atherosclerosis	Flow Cytometry	[85]
	White blood cell count (WBC)	WBC count is an indicator of systemic inflammation. Augmented WBC count predicts cardiovascular mortality.	Mortality, CVD	Automated Blood Cell (Coulter) Counter	[86]
Inflammatory Function	C-reactive protein (CRP)	Protein which is related with inflammation. CRP levels are increased in the brains of AD patients.	Stroke, CVD, Heart attack, Arthritis, Dementia	Immuno-turbidimetric Assay	[87]
	Interleukin-6 (IL-6)	Cytokine that answers to acute diseases. Strong predictor of mortality and immune aging illnesses. The IL-6 levels are higher in older people.	CVD, AD, Immune disorders, Diabetes mellitus	RIA	[88]
	Tumor necrosis factor-α (TNF-α)	Pro-inflammatory cytokine that has a crucial role in the pathogenesis of chronic inflammatory diseases.	Diabetes, Arthritis, Stroke, Obesity, Atherosclerosis	ELISA	[89]
	Fibrinogen	Protein involved in coagulation process.	Mortality, CVD, AD	Clauss Method, kinetic Fibrinogen Assay	[90]
	Albumin	The main protein produced by the liver which aids maintains osmotic pressure and does the transport of small molecules for blood. Lower serum albumin levels are often present in elderly.	Heart attack, Stroke	Quantification using Gornall, Bardwill and David Method	[91]

Domain: Biomarkers of Nervous System					
Central Nervous System (CNS)	Amyloid β42 (Aβ42)	One of the constituents of the senile plaques. Secretases cleaved the Aβ from the large amyloid-precursor protein (APP) and in amyloidogenic pathways yields a 42 amino acid peptide that can aggregate in the brain. Amyloid β peptide is responsible too for oxidative damage.	AD, Frontotemporal dementia	ELISA	[92]
	Total Tau (t-Tau)	A component of neurofibrillary tangles. Total tau levels are considerably higher in AD and Creutzfeldt-Jakob disease patients.	AD, Creutzfeldt-Jakob disease	ELISA	[93,94]
	Phosphorylated Tau (pTau)	Tau is hyperphosphorylated in AD, which the consequence is a lack of function and axonal transport dysfunction. The presence of tau phosphorylated at position -181 is expressively increased in AD. Phosphorylated forms of tau (phospho-tau -181, -199, -231, -235, -396 and -404) may give important progresses in early diagnosis of AD.	AD, MI	Immuno-precipitation, Immunoblotting	[95]
	F_2-isoprostanes (F_2-IsoP)	Isomer of prostaglandins (PG) resultant from oxygen radical peroxidation of arachidonic acid esterified in membranes of the cells. Enhanced levels of 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) are described in AD brain and amyloid-peptide stimulates the production of 8-iso-$PGF_{2\alpha}$.	AD, Atherosclerosis plaque, High cholesterol levels	Isotope Dilution (Free F_2-IsoP in 1 to 2 ml of CSF), GC/NICIMS	[96]
Sympathetic Nervous System (SNS)	Norepinephrine (noradrenaline)	Norepinephrine is a catecholamine with multiple functions as a hormone and a neurotransmitter. Indicator of stress answer. Synthesized from dopamine	Mortality CHF, MI,	Tritiated (-)-Noradrenaline Analysis	[97]
	Epinephrine (adrenaline)	Hormone secreted by adrenal medulla. Strong emotions like anger or fear lead to the release of epinephrine in the bloodstream,	Cognitive decline, MI	Isotope Dilution (radiolabeled epinephrine)	[98]

		causing an increase in heart rate, muscle strength, blood pressure, and sugar metabolism - "flight or fight response" – which prepare the body for strenuous activity.			
Domain: Biomarkers of Molecular Mechanisms					
Oxidative Stress	Reactive oxidative species (ROS)	Increases during lifespan. Causes enzyme inactivation, deterioration of membranes and altered cellular function.	Parkinson's disease, DNA damage	ESR Spectroscopy	[99]
	Superoxide dismutase (SOD)	Antioxidant functions in cells which are exposed to oxygen.	Inverse relation to AD, Hypertension	Enzymatic Assay according to Marklund and Marklund Method	[100]
	8-hydroxy-2-deoxyguanosine (8-OHdG)	Oxidative damage of nucleic acid. The major product of DNA oxidation. Elderly have higher levels of 8-OHdG	AD, Arthritis, Atherosclerosis, Cataracts, Hypertension, Osteoporosis, Type II diabetes	Urinary Enzyme-Linked Immunosorbent Assay	[101]
	8-Isoprostane	Phospholipid oxidative damage. 8-Isoprostane is produced by non-enzymatic oxidation of arachidonic acid present in phospholipid membranes. Higher concentration levels are present in elderly.	Arthritis, Cataracts, Hypertension, Type II diabetes	Enzyme Immunoassay	[102]
	Glutathione (GSH)	Decreases with aging and in some ARD. GSH has the function to protect the cells from oxidative stress.	AD	Enzymatic Assay in brain	[103]
	Serum Hsp70 (Hsp – heat shock proteins)	Hsp70 declines in elderly.	AD, Parkinson's disease, Huntington's disease, Ataxias, Amyotrophic lateral sclerosis	ELISA	[104]
	Tyrosine	Protein oxidative damage. Tyrosine is created from phenylalanine. The attack of ROS on phenylalanine form o-tyrosine and m-tyrosine. Elderly have decreased levels of o-tyrosine.	Cataracts, Type II diabetes	LC (serum)	[105]
Genomic Instability	Chromosome structural aberrations levels	During aging occurs a significant accumulation of genetic damages, mainly deletions, translocations and chromatid breaks.	Several diseases	Cytogenetic Analysis	[106]
	DNA repair activity	Mechanism to correct chemical damages and breaks in the DNA, this	Several diseases	Enzymatic Assay	[107] Reviewed in [108]

		process is realized by specific enzyme systems. DNA repair decreases with aging.			
	$p16^{INK4A}$ expression level	Protein involved in the cell cycle control. The cell cycle arrest can leads to senescence, apoptosis or turns normal cells in a cancer cells. $p16^{INK4A}$ expression can promote aging and prevent cancer.	Several diseases	Flow Cytometry, Western Blotting, Immuno-histochemistry in lymphocytes	[109]
	p21 expression level	Inhibits the cyclin-dependent kinase. Has an important role in DNA damage answer and regulates the cell cycle. p21 is an effector of the functions of p53 which promotes senescence. p21 has a dual role in aging, since it can promote or prevent aging.	Several diseases	Western Blotting, qRT-PCR	[110]
	p53 expression level	Transcription factor which controls the cell cycle. Is involved in repair process and DNA damage identification. p53 is tumor suppressor and is noticeable in aging cells.	Several diseases	Western Blotting, qRT-PCR	[111]
	Level p53-binding protein 1 (53BP1)	53BP1 binds to p53 protein implied in DNA damage response. The manifestation of an increase in 53BP1 foci is a dependable biomarker of aging.	Several diseases	Immuno-fluorescence, FISH, TEM	[112]
	Number of deletions in mitochondrial DNA	With the age the number of mitochondria reduces due to their diminished proliferation and death, caused by mutation in mtDNA.	Several diseases	qPCR, Dot Blot	[113]
Senescence	Level of senescence-associated β-galactosidase (SA-β-gal)	SA-β-gal accumulates over the aging process and its activity is enhanced in old cells.	Several diseases	Histochemical Analysis of brain and Cytochemical Analysis of hippocampal neurons	[114]
	Apoptosis levels under the impact of hydrogen peroxide	Fibroblasts of aged animals present higher levels of apoptosis after damage with reactive oxygen species than young animals.	Several diseases	Flow Cytometry	Reviewed in [115]
	Number of senescent cells	Cells that lose their proliferative capacity. The agglomeration of some quantity of senescent cells is enough to beginning the aging process in the tissues, organs or in the entire organism.	Several diseases	Immuno-fluorescence, Immuno-FISH	[116]

	Ink4a/Arf	The Ink4a/Arf locus codifies two tumor suppressor molecules, Arf and $p16^{INK4a}$, the main mediators of senescence in cells. $p16^{INK4a}$ and Arf increase in the majority tissues with aging.	Several diseases	qRT-PCR	[117]
Epigenetic Alterations	γ-H2AX foci number	γ-H2AX is an indicator of conformational chromatin changes and double-strand-breaks in DNA. High quantity of γ-H2AX foci is observed in nuclei of aging cells.	Several diseases	Immuno-histochemistry	[118]
	Trimethylated lysine at the 27 position of histone H3 (H3K27me3)	DNA methylation levels are diminished in aging cells. H3K27me3 levels decrease with age and in accelerating aging processes.	Several diseases	Western Blotting, Immuno-histochemistry	[119]
	Trimethylated lysine at the 9 position of histone H3 (H3K9me3)	Is a marker of constitutive chromatin and their levels decrease with aging and in premature aging processes.	Several diseases	Immuno-fluorescence, Immunoblotting, FISH	[120]
	Number of senescence-associated heterochromatin foci (SAHF)	Domain of facultative heterochromatin. Its formation happens with aging and their mechanism describes the irreversibility of the cell cycle arrest in aging.	Several diseases	Immuno-fluorescence, FISH	[121]
Telomere Attrition	Telomere length	Telomeres become smaller at each cell division. Telomere length is responsible for several age- associated diseases.	Several diseases	Immuno-FISH	[122]
	Telomerase activity	Enzyme that extends the ends of telomeres. The number of cell divisions is directly dependent of telomerase. Telomerase activity decreases during aging	Several diseases	RT-PCR	[123]
Others	Shape of nuclear lamina	Basis of the nuclear membrane. Cells with uncommon form of the nuclei accumulate during aging.	Several diseases	Immuno-fluorescence	[124]
	Progerin level	During aging there is an accumulation of progerin.	Several diseases	qRT-PCR	[125]
	Wound healing rate	Mechanism of damage repair of tissues. Wound heal is more sluggish as we age.	Several diseases	Mathematical method	[126]

AD: Alzheimer's disease; CAD: coronary artery disease; CHD: coronary heart disease; CHF: congestive heart failure; COPD: chronic obstructive pulmonary disease; CSF: cerebrospinal fluid; CVD: cardiovascular disease; ELISA: enzyme-linked immunosorbent assay; ESR: electron spin resonance spectroscopy; FISH: fluorescence *in situ* hybridization; GC: gas chromatography; GC/NICIMS: gas chromatography/negative ion chemical ionization mass spectrometry; HPLC: high performance liquid chromatography; LC: liquid chromatography; MI: myocardial infarct; qPCR: quantitative polymerase chain reaction; qRT-PCR: quantitative real-time polymerase chain reaction; RIA: radioimmunoassay, RT-PCR: real time polymerase chain reaction and TEM: transmission electron microscopy.

6. THE POWER OF -OMICS IN THE STUDY OF AGING

Aging research needs multi and transdisciplinary methods using novel high throughput technologies that are persistently in development, enhancing exponentially the biological information about aging process and clarifying uncharted mechanisms [56]. Ideally, a systematic approach for biomarker identification will involve multiple technologies to investigate the aging process at all levels. Thus, for a complete approach of aging, an "-omics" approach is needed. Genomics is used to identify relevant disease genes, aberrant cellular signaling pathways, and expression signatures correlated with aging. Proteomics is used to study protein translation, inclusive post-translational modifications and can be used to identify aberrant protein expression. Transcriptomics includes the measurement of mRNA levels for quantifying gene expression. And finally metabolomics is useful to identify the presence of abnormal levels of small molecule metabolites that are specific to and indicative of an underlying aging process [39,136–138].

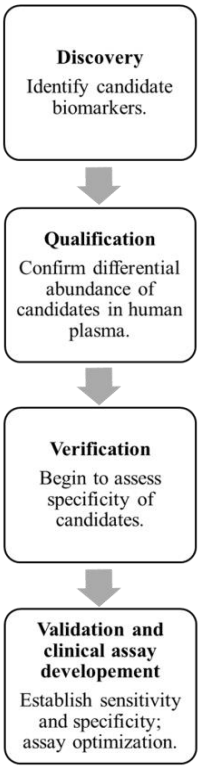

Figure 2 - Different steps of biomarker development. Adapted from [129].

Biomarker discovery tends to take one of two options: target approach, which is designed to investigate a specific molecule or group of molecules and non-targeted approach (metabolic profiling and metabolic fingerprinting) designed to analyze a large number of molecules in an unbiased manner. Within targeted approaches, commonly used techniques are nuclear magnetic resonance (NMR) and magnetic resonance spectroscopy (MRS), multiple mass spectrometry (MS)-

based methods, and immunoassays (e.g. ELISA). Since there is an increasing trend for biological samples to be analyzed without a specific target molecule, within non-targeted approaches metabonomics and metabolomics fields have been expanding [139].

Metabolite profiling restricts itself to a certain range of compounds or even to screening a pre-defined number of members of a compound class. For these two approaches, main techniques are gas chromatography (GC), high performance liquid chromatography (HPLC) and NMR [140]. Metabolic fingerprinting does not attempt to identify or precisely quantify all the metabolites in the sample. Rather, it considers a total profile, or fingerprint, as a unique pattern characterizing a snapshot of the metabolism in a particular cell line or tissue. The metabolic fingerprinting is very useful in biomarker discovery and diagnostic, namely to discover specific metabolic patterns of diseases.

Mass spectrometry (MS) and Fourier transform infrared (FTIR) spectroscopy are usually used in fingerprinting. While MS is often associated with separation techniques (LC, GC or SDS-PAGE) FTIR may not require *prior* separation of the components of the sample [141]. Proteomic technologies introduced the concept of a blood signature, arguing that a combination of plasma biomarkers (i.e. a signature) rather than a single marker will be needed to reflect the complexity of aging [136]. Besides, proteomics has garnered recent attention mainly because proteins are readily available in body fluids and are more stable than mRNA and metabolites and because it allows to study both structure and function of proteins by several methods, whether qualitative or quantitative, allowing to reveal static or perturbation-induced changes in a protein profile [142]. Proteomics provides an unbiased approach to detecting protein biomarkers in complex sample mixtures such as plasma. It uses MS data coupled with protein database pattern recognition search algorithms to identify large numbers of proteins simultaneously. One of the most powerful protein identification strategies is liquid chromatography (LC) separation followed by MS analysis, usually using ESI (electrospray ionization). Few studies have used proteomic methods to discover new AD plasma biomarkers, making neuroproteomics an emerging technology. Plasma proteins that have been identified by these techniques are related with lipid or other molecules transportation, immune regulation and inflammation [143]. Moreover, it has been developed a new field called peptidomics, a proteomic analysis of peptides and small proteins, since proteins can be cleaved aberrantly (e.g. APP cleavage by α and β- secretases). So direct *de novo* analysis of endogenous peptides could be invaluable for sample characterization and in some cases disease diagnosis [142].

Although being still a relatively recent field, metabolomics is being developed and applied as

a more global approach to several age-related disorders biomarkers discovery. Metabolomics is now commonly defined as the –omic to detect and quantify all low-molecular-weight molecules and metabolites present in cells, tissues or organisms under a set of given conditions. Thus, it is able to quantify metabolite changes indicative of alterations in anabolic and catabolic processes in the body. In turn, metabonomics is described as the quantitative analysis of metabolic response of living systems during pathophysiological stimuli or genetic modification over time [136,137]. Metabolomics is typically performed on biofluids, such as serum, plasma, urine, saliva and CSF [138]. By assessing hundreds of metabolites simultaneously, metabolomic techniques produce high-resolution biochemical snapshots showing the functional endpoints of genetic predisposition as well as the sum of all environmental influences such as nutrition and medication. This snapshot can provide an almost real-time image of the pathophysiology of an entire organism. This information it is not possible to obtain in genomics, proteomics or in other "-omics" [144]. Specifically, due to the fact that any biochemical change must precede any morphological manifestation of the disease itself, a metabolomic approach may be very useful for early diagnosis. It requires analytical techniques of analysis such as chromatography, molecular spectroscopy or mass spectrometry, coupled with bioinformatics tools such as multivariate data analysis methods to get the most out of the data. Thus, metabolomics information complements data obtained from genomics, transcriptomics and proteomics [145]. Metabolomic techniques have been used to identify biochemical pathways' perturbations related to several diseases, either in biological fluids (plasma, urine and CSF), animal models and tissue or cell cultures [145]. A typical metabolomics study includes four main steps: uniform sample collection, sample analysis (e.g. NMR, MS, FTIR), statistical analysis (e.g. PCA, PLS-DA), and identification of significantly altered metabolites that could be translated into new disease biomarkers/pathways [146]. The main metabolic fingerprinting approaches used for disease diagnostics includes infrared (IR) and Raman spectroscopy, NMR and MS-based methods. Hence the multiple advantages and more widespread use of infrared (IR) spectroscopy, its role in metabolomics will be emphasized ahead [137]. One of the main metabolomics challenges is the development of standard protocols regarding sample collection and storage, chemical analyses, data processing, and information exchange. Indeed, it was already created the Metabolomics Standard Initiative, with support of the Metabolomic Society, to recommend standard protocols for use in all aspects of metabolomics research [145].

6.1. Infrared Spectroscopy

Spectroscopy is increasingly become one of the main tools for biomedical applications and has made noteworthy progress in the field of clinical evaluation [147]. The infrared spectroscopy is a powerful analytical technique based on the vibrations of the atoms of a molecule. This method is one of the most ordinary and used spectroscopic techniques due to its effectiveness in provide information about the purity of different compounds and identifying its structures allowing knowledge of functional groups, bonding types and molecular conformations [147–149]. Nucleic acids, lipids, carbohydrates, the primary and second structure conformation of proteins, body fluids, tissues and cell cultures are some of the diverse compounds that can be analyzed by IR spectroscopy [147,148]. An infrared spectrum is obtained by IR spectrometer device when the infrared radiation (restricted in electromagnetic spectrum between the visible and microwave regions – figure 3) crosses through a sample. Each peak of energy present in the absorption spectrum matches to the frequency of a vibration of a part of a molecule of the sample. Molecular rotations and molecular vibrations are the motions of the atoms in a molecule. The vibrational states of the atoms are the responsible for allow infrared radiation absorptions so that the IR spectrum is a vibrational spectrum where is possible observe quite sharp bands [148,149].

The infrared radiation is subdivided into three different radiation types based on the wavelength: near-infrared (NIR) (14000 to 4000 cm^{-1}), mid-infrared (MIR) (4000 to 400 cm^{-1}) and far-infrared (FIR) (400 to 4 cm^{-1}) (figure 3). The MIR region is unique for a molecule which normally provides information about fingerprint characteristics of molecular species and is divided into four regions: X–H stretching region (4000–2500 cm^{-1}), the triple-bond region (2500–2000 cm^{-1}), the double-bond region (2000–1500 cm^{-1}) and the fingerprint region (1500–600 cm^{-1}) [148]. In sum, when the radiation absorbed has equal frequency of the molecular vibration and the dipole moment of a molecule is altered due to molecular vibration, IR radiation is absorbed leading to the transition of a molecule. The transitions may occur from the v=0, ground vibrational state, to v=1, first excited vibrational state (this process is called fundamental tone) or from v=0 to v=2, v=3, v=4…, which is a process denominated overtone. In this process, these absorptions are weaker than in fundamental tone. Thus, in infrared absorbance spectroscopy we measure the quantity of infrared radiation transmitted to a sample in a range of frequencies of electromagnetic spectrum, being molecular spectroscopy an evaluation method of the spectra formed by the interaction amongst a sample and radiation [149–151].

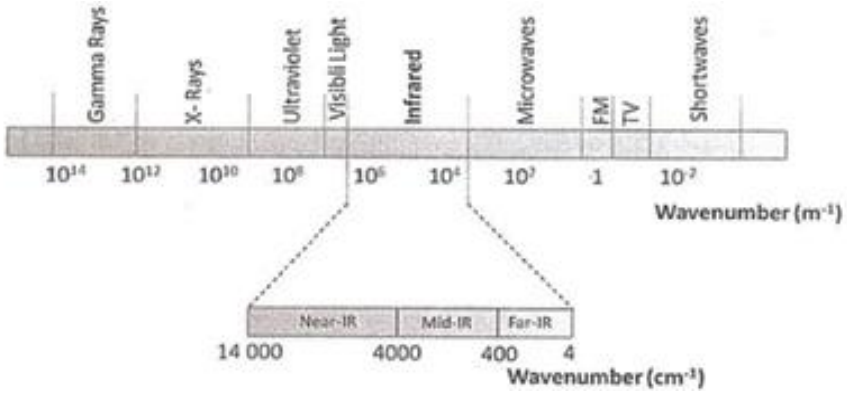

Figure 3 - The electromagnetic spectrum.

IR spectroscopy is a rapid, non-destructive easy to use tool which allows measurements at minimal costs making possible the monitoring of diseases, opening doors for personalized medicine [147–149,152].

In an IR spectrum the abscissa axis corresponds to the wavelength, wavenumber or frequency and the ordinate axis to the absorption intensity (absorbance - A) or to the percentage of transmittance (transmittance - T). The main goal for the elaboration of a spectrum is determine a signal (S), which represents the amount of radiation absorbed, emitted or reflected by the sample. When a signal is obtained is also obtained a noise (N) which may be originated from diverse sources. To assess the quality of an IR spectrum the signal-to-noise ratio (SNR) is quantified and the quality of the spectrum is greater the higher SNR is [149].

In general, in spectra analysis, 100% of transmittance corresponds to zero absorption of light at that wavelength. Absorbance is mostly used for quantitative work and multivariate analysis while the transmittance is commonly used for spectrum interpretation. However, it arises from personal preference the choice of which method to use. The wavenumbers and the complexity of the peaks in the spectrum provide data about the molecules. The complexity is advantageous due to combine an experimental spectrum with a known compound and the frequencies or wavenumbers give information about the functional groups existing in the molecule. Therefore for the spectra analysis it is essential the help of tables which correlate frequencies with functional groups and the theoretical and experimental spectra [148,153,154].

6.1.1. FTIR as a Useful Metabolic Fingerprint Tool

Since the beginning of 1950s, IR spectroscopy has been an important technique for materials analysis in the laboratory. Fourier Transform Infrared (FTIR) spectroscopy is now the main method of IR spectroscopy, available for routine work in laboratories.

FTIR is a technique almost universal because many molecules have strong absorbances in the mid-infrared region, and is applicable for many types of samples (solids, liquids, gases and pasts). It is highly versatile, since is needed minimum sample preparation. Infrared spectra are rich in information, wherein peaks positions give the information about the structure of molecules in a sample. The intensity of peaks is related to the concentration of molecules and peak widths are sensitive to the chemical matrix of the sample (pH and hydrogen bonding).

Given that FTIR spectroscopy is a technique based on metabolomics, it is able to detect biochemical changes caused by pathologies, even at a very early stage of the disease. In addition, comparing with other laboratory instruments (e.g. for NMR, GC-MS or LC-MS) infrared spectrometer is relatively inexpensive [155]. It gives the opportunity to analyze, in real time, small amount of sample, providing reliable and reproducible spectral data [156]. Whilst it is not as specific and sensitive as other techniques of metabolomics such as GC/TOF-MS, it has been recognized as a valuable tool for metabolic fingerprinting, owning to its simplicity and ability to analyze several biomolecules simultaneously [157].

FTIR can enhance speed collecting a scan each second, it is a precise measurement method which does not need external calibration and has highly sensitivity and specificity [158,159]. This new approach of spectroscopy has valuable applications in several fields of biomedical research and in specific aging research and can be productively applied to samples from a huge variety of cell lines, blood cells and tissues. The outcomes of many research studies have concluded that FTIR together with the use of proper statistical data analysis techniques has a strong accurateness in identifying and classifying chemical and pathophysiological changes in aging and the different biomolecules in successful and unsuccessful aging, providing a rapid and non-invasive diagnostic test [147,160]. Although FTIR has been recognized as not as specific and sensitive as some of the MS-based techniques, the rapidity and reproducibility of FTIR is demonstrable through the extensive number of published research using this technology. Besides, it has been recognized as a valuable tool for metabolic fingerprinting as it is able to analyze carbohydrates, amino acids, fatty acids, lipids, proteins, nucleic acids and polysaccharides simultaneously with a minimum amount of sample preparation. Indeed, besides its rapidity (<10 seconds *per* sample is readily achievable), FTIR is an ideal candidate technology for high-throughput screening as it is possible to measure thousands of samples *per* day and obtain information-rich spectra using only 0,5µL of sample. Also, FTIR is less expensive and more accurate rather other methods [137,161].

6.1.2. Potential of the FTIR in Diagnosis of Age-Related Diseases

FTIR has been applied to samples of human tissue and body fluids and has shown potential to support clinical pathology, i.e. detecting and grading of disease [162,163]. In this context FTIR spectroscopy proved to have potential in cancer diagnosis [150,164,165] such as cervical cancer [166], colorectal cancer [167], liver cancer [168], prostate cancer [169,170] and breast cancer [171]. The diagnosis and evaluation of the chemotherapeutic efficacy of patients with leukemia was also achieved with this technique [172,173]. Others applications include diagnosing arthritic disorders [174,175] and determination of kidney complex stones composition [176,177]. Infrared spectroscopy has also proved to be a valuable tool for characterizing and differentiating microbial cells [178], proving a rapid method for identifying microorganisms responsible for human infections [179]. In addition, FTIR has proved to be very useful to understand the mechanism of atherosclerosis development [180,181] and the diabetes diagnosis [182,183]. FTIR has been used in several pathology research fields besides neurodegenerative diseases [184]. With particular relevance, FTIR spectroscopy is used in various studies and in diagnosis of aging-related diseases such as the study of protein misfolding and aggregation in Alzheimer's, Parkinson's and Huntington's diseases and in prion disease Creutzfeldt–Jakob [185]. FTIR is called the green analytical method and is used to quantify the levels of choline (nutrient) and its metabolite betaine, which are metabolic associated to trans-methylation pathways including synthesis of total homocysteine, a risk factor of CVD [186]. In atherosclerosis, FTIR is used to discriminate biochemically and structurally the different depositions of atherosclerotic plaques [187]. In the context of diabetes, FTIR spectroscopy has been currently used in research studies however it has not yet been applied in clinical practice. Notwithstanding the crucial aim is to quantify the levels of HbA1c to allow the screening and assessment of diabetes in patients [188]. Relatively to arthritis FTIR is used to distinguish healthy and osteoarthritic cartilages [189]. In the future, it is expected that FTIR, in association with other spectroscopic methods, will continue to be used in order to conduce to a better comprehension, diagnosis and treatment of aging and aging-related diseases [185].

7. CONCLUSION

The aging population is the source of huge challenges to healthcare provision and treatment of ARD. The study of aging biomarkers might help to comprehend in a molecular dimension the aging physiology. Ideally, biomarkers of aging should assess the biological process of aging and not the presence of ARD. Regrettably, elderly population typically presents several co-morbidities

that challenging the identification of precise biomarkers and it is impossible to study a human being during their entire lifespan in order to detect biomolecules that arise and are specific of aging. Therefore, aging biomarkers are urgently necessary to evaluate the health state of aged people and the potential therapeutic interventions. In the study of aging and age-related diseases, it is essential to use reliable methodology of analysis that reflects the changes occurring during the aging process and that can differentiate healthy elderly people from elderly people with age-related diseases and clarify which biomolecules give an aged condition, using peripheral fluids and implying reduced costs.

The absorption of radiation in the mid-infrared spectral region provides itemized information on the chemical composition of biological samples and, such the majority of molecular species absorbs infrared light, giving rise to distinctive spectral patterns in transmitted light, it is possible to use FTIR as a potential method to study the physiological and pathological conditions which are related with homeostatic alterations of cells and tissues. This technique allows identifying aging-related signals at a biochemical level, helping to identify specific biomarkers. Plasma is a reliable biofluid for metabolic studies containing numerous biomarkers that can be studied by FTIR. The FTIR spectral acquisition and multivariate analysis will help to confirm biomarkers previously identified through FTIR spectroscopic assignment and possible discovery and develop novel aging biomarkers, helping to better understand the aging process and metabolic aging physiological modifications.

8. SIGNIFICANCE OF THE STUDY

Aging is an unavoidable physiological alteration occurring in organisms over time. Ultimately, it leads to death due to successive dysfunctions in every organs of the organism. [25]. Aging is a critical risk factor for several human diseases like neurodegenerative and metabolic diseases and many types of cancer which have become much more prevalent in the elderly. It is a fact that world's population is aging. In order to improve healthspan of elderly and increase lifespan there are a greater need to understand the mechanisms associated with aging process. It is known that, many of the neurodegenerative diseases are related with protein aggregates. However, the major question which is: what is the relationship between protein aggregates and physiological aging? With advancing age there is a loss of proteostasis, being one of the nine hallmarks of aging. In an attempt to understand the correlation between aging, protein changes and the presence of protein aggregates several studies have been developed.

The main goals of this study are:

1 Evaluate the ability of FTIR to identify protein aggregates/alteration in protein conformation in cell models (yeasts);
2 Correlate aging with the presence of protein aggregates/alteration in protein conformation in human plasma samples;
3 Contribute to the identification of biological predictors of aging particular the presence of protein aggregates/alteration in protein conformation by FTIR.

Chapter Two

This chapter gives an overview of Fourier Transform Infrared spectroscopy principles and of FTIR equipment and functioning, as well as sample characterization and a description of the methods used in this study.

2. FTIR METHOD OVERVIEW

2.1. Fundamental Principles

Fourier Transform Infrared spectroscopy is a technique that can be applied to a wide range of samples and it is based on the analysis of the interaction of IR light with a substance. FTIR spectroscopy constitutes the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. Some of this radiation is absorbed by the sample, due to the vibrations of chemical bonds of the molecules, and some of it is transmitted. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the analyzed sample. In an IR spectrum it is possible to identify sample's functional groups and/or elucidate its molecular structure (qualitative analysis) through the absorption peaks, besides, the size of these peaks are directly related with the quantity of each functional group (quantitative analysis). FTIR spectrometers are used for acquisition of infrared spectra and these devices are able to analyze different types of samples such as solids, liquids and gases [148,190].

2.2. Molecular Vibrations

The molecules are not static, when they interact with radiation they oscillate/vibrate. The energy possessed by a molecule at any moment is defined as the sum of vibrational energy which corresponds to the absorption of energy by a molecule as atoms vibrate about the mean center of their chemical bonds; translational energy, which is correlated to the molecules displacement in space as a function of the normal thermal motions of matter; electronic energy, which is linked to energy transitions of electrons as they are distributed in the molecule and rotational energy, the tumbling motion of a molecule being the result of energy absorption within the microwave region [191]. The IR radiation does not has enough energy to induce electronic transitions as ultraviolet (UV) radiation, thus the absorption of IR is limited to compounds with small energy differences in the rotational and vibrational states. When a molecule interacts with infrared radiation there will be changes in molecular dipoles associated with rotations and vibrations, the larger the dipole change, the stronger the band intensity in IR spectrum [148,192,193].

A molecule in its resting stable energy state has a number of freedom degrees which represents the potential to change position in space. N is the number of atoms in a molecule, so a molecule containing N atoms has 3N degrees of freedom which corresponds to motions along any Cartesian coordinates axes (x, y, z). So, each atom has three degrees of freedom and polyatomic molecules of N atoms have 3N total degrees of freedom. Nonetheless, three degrees of freedom are necessary to describe translational, and other three correspond to rotation of the whole

molecule. Thus, polyatomic non-linear molecules have 3N-6 degrees of freedom (three translational and three rotational) and polyatomic linear molecules have 3N-5 degrees of freedom (three translational and two rotational). The remaining 3N-6 and 3N-5 correspond to fundamental vibrations [194].

The atomic positions in molecules are not fixed, once they are subject to several vibrations related with changes in bond angle or bond length. The main types of molecular vibrations (figure 4) are stretching (axial deformation): change in inter-atomic distance along bond axis, which presents two different types: symmetric stretching and asymmetric stretching (asymmetric stretching happen at higher frequencies than symmetric stretching) and bending (angular deformation): change in angle between two bonds, which has four distinct types: rocking, scissoring, wagging and twisting. Bending vibrations take place at inferior frequencies than stretching vibrations [148,192,193].

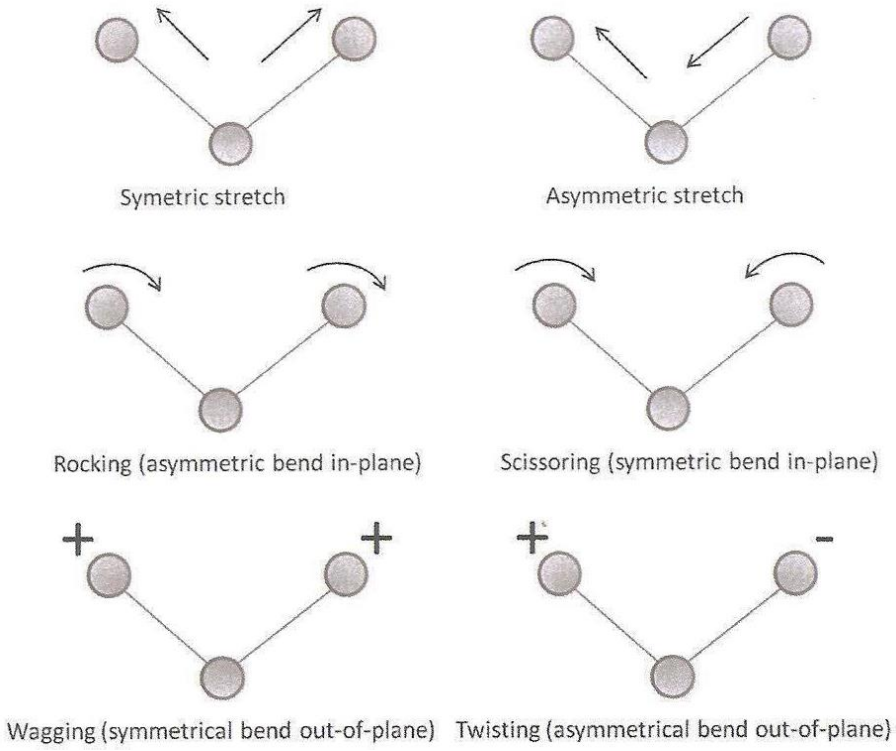

Figure 4 - The different vibrational modes of molecules, divided on stretching type and bending type (in-plane and out-of-plane) Adapted from [148].

Isolate the motion of two atoms in one molecule from the motion of the rest of the atoms in a molecule is not conceivable. One atom might be mutually shared by two distinct oscillating bonds and when this occurs it is designated that the vibrations of the two bonds are coupled. So, vibrational coupling is influenced by several factors: strong coupling of stretching vibrations

happens when there is a common atom among two vibrating bonds; bending vibrations coupling occurs if there is a common bond between vibrating groups; coupling between bending vibration and stretching vibration occurs if the stretching bond of one side of an angle varies by bending vibration; when coupled groups have roughly equal energies the coupling is greatest; there is no coupling amongst groups separated by two or more bonds [192,193].

2.3. FTIR Spectrometers

Nowadays, Fourier Transform Infrared spectrometers (figure 5) are largely used and have improved the acquisition of IR spectra. Its basic components are: the radiation source, the interferometer (predominantly Michelson interferometers), the sample, the detector and a computer to process the data. FTIR spectroscopy is based on the idea of the interference of radiation among two beams to create an interferogram, which is a signal produced in function of the alteration of the path length between the two beams. The two domains of frequency and distance are interconvertible by the mathematical method of Fourier Transform (FT) [148].

The IR radiation is emitted from the source and passes through an interferometer to the sample before getting the detector. The interferometer has in its center a semi-reflecting film, the beamsplitter, which bisects the planes of the two mirrors presents. The Michelson interferometers are constituted by two perpendicularly plane mirrors, one of them is a fixed flat mirror and the other one is a moving flat mirror which allows moving this mirror a very short distance away from the beamsplitter. Whenever a parallel beam of radiation passes through an ideal beamsplitter 50% of the incident radiation will be reflected to one of the mirrors whilst the remaining 50% will be transmitted to the other mirror. Thereafter the radiation reflected from the mirrors returns to the beamsplitter to recombine and interfere. Through the beamsplitter is transmitted 50% of the beam reflected from the fixed mirror already the other 50% is reflected back in the direction of the source. The moving mirror in the interferometer generates an optical path difference among the two arms of the interferometer and it produces constructive interferences, in the case of the reflected beam, and destructive interferences, in the case of the transmitted beam. The subsequent interference pattern is designated an interferogram.

As the interferogram is measured, all frequencies are measured simultaneously, resulting in an extremely fast IR measurement so this interferogram signal cannot be interpreted directly, since it is necessary a frequency spectrum (a plot of the intensity at each individual frequency). Thus, the FT method, performed by a computer, will “decode” the individual frequencies, converting the interferogram in an infrared single beam spectrum and the number of peaks

present will represent the number of detectable constituents of the sample. It is also essential measure a background spectrum, since it is required to has a relative scale for absorption intensity [148,150,190].

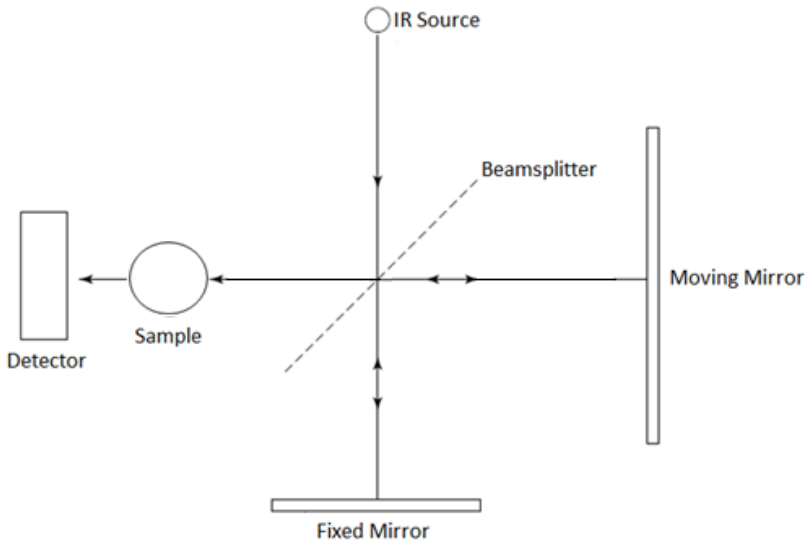

Figure 5 - Schematic representation of Fourier Transform spectrometer and its main components. Adapted from [148].

2.4. Attenuated Total Reflectance (ATR) Spectroscopy

The reflectance techniques might be used for samples that are complicated to analyze through the conventional transmittance methods. Attenuated Total Reflectance (ATR) technique revolutionized solid and liquid FTIR analysis once it tries to solve the most important challenging aspects of IR analysis: sample preparation and spectral acquisition. ATR spectroscopy provides faster sampling, improves sample-to-sample reproducibility and minimizes user to user spectral variation [148,195].

ATR uses the phenomenon of total internal reflection, so ATR device measures the changes in a totally internally reflected IR beam when the beam comes in contact with a sample. The beam of radiation entering in the ATR crystal will undergo total internal reflection when the angle of incidence at the interface among the sample and crystal is greater than the critical angle. This internal reflectance originates an evanescent wave which will be attenuated or altered in regions of IR spectrum where the sample absorbs energy. The resultant attenuated radiation is measured and plotted as a function of wavelength through the spectrometer and gives rise to the absorption spectral characteristics of the sample [148,194,195].

ATR spectroscopy is a technique with a lot of advantages, once requires little or no sample preparation for the majority of the samples, improves spectral acquisition and reproducibility

leading to higher quality in database building, more precise material identification and verification and is considered one of the most versatile sampling techniques [194,195].

2.5. FITR Advantages

FTIR instruments have several advantages over the traditional dispersive technique including:

- Increased speed: all frequencies are measured simultaneously (Felgett advantage);
- Improved sensitivity: once the detectors are considerably more sensitive and the optical throughput is greater resulting in much lower noise levels (Jacquinot advantage);
- Faster scans allow the co-addition of many scans in order to reduce the random measurement noise to any desired level (signal averaging);
- Mechanically simple: since it contains only a moving part, the interferometer moving mirror is the unique continuously moving part in the FTIR device;
- Internally calibrated: FTIR instruments employ a HeNe laser as an internal wavelength calibration standard (Connes advantage) becoming self-calibrating. So this technique provides a precise measurement which no requires external calibration;
- Non-destructive technique [190].

The choice of this technique in this work is related with the fact that has low costs associated, is a non-destructive technique and the sample analysis is very fast allowing having the results in a short period of time.

3. METHODS OF STUDY

3.1. Sample Collection

Human plasma is commonly used in biological and clinical studies. To acquire it, it is necessary to add an anticoagulant such as EDTA (ethylenediamine tetraacetic acid) or heparin in the blood collected samples, following by removal of blood cells [196]. Optimizations were performed in the sample collection method in order to obtain samples with high quality and quantity and to overcome water signals overlap in IR spectra. Since water is the most abundant compound of all biological fluids and has a strong absorption in MIR region this becomes a problem when FTIR is used. In order to avoid water spectral signals drying process was used [197].

In the present study, plasma aliquots of the samples were obtained using K_2EDTA tubes (5 mL) with gel separator and then it was applied a centrifugal force of 1800g during 15 minutes at 4ºC, in which the blood cells were separated from plasma. Were performed plasma aliquots of 30µL for the present study which were stored at - 80ºC.

3.2. Characterization of Human Plasma Samples

The inclusion criteria for the present study group were: age over 65 years, resident in Aveiro, with no comorbidities or memory impairment. The exclusion criteria include individuals which were undergoing radiotherapy or chemotherapy, suffering from psychiatric illness (e.g. schizophrenia, bipolar disease) or using illicit drugs.

To evaluate the cognitive status of the selected individuals was carried out at several centers for primary health cares from Aveiro region the following cognitive tests: CDR (clinical dementia rating scale), MMSE (mini-mental state examination) and GDS (geriatric depression scale). This final test allowed the exclusion from the study of depressed individuals.

A project to distinguish Alzheimer's disease patients from control patients (with no AD) was approved by the ethics committee of the Regional Health Centre – Coimbra, protocol number 012 804 of April 4, 2012. However in this present study only the control samples of the approved project were used, presenting negative results for all cognitive tests (CDR -; MMSE -; GDS -) (table 2).

Table 2 - Characterization of the study samples according to gender, age and results of cognitive tests.

COD	Sex	Age	MMSE	CDR
C1	F	65	-	-
C2	F	65	-	-
C3	F	65	-	-
C4	F	75	-	-
C5	F	75	-	-
C6	F	75	-	-
C7	M	74	-	-
C8	M	78	-	-
C9	M	82	-	-
C10	M	82	-	-

3.3. Experimental Procedure for Yeasts

The strains of *Saccharomyces cerevisiae* used in this study were kindly provided by RNA Biology laboratory of iBiMED – Institute of Biomedicine, University of Aveiro (Manuel Santos and Joana Tavares).

We used two different strains: a wild-type strain and a knockout of a specific tRNA modifying enzyme, which causes extensive protein aggregation. Protein aggregation was measured by fluorescence microscopy since all strains have a fluorescent molecular sensor that allows seeing foci of aggregated proteins in the cell. The percentage of protein aggregation in wild-type strains

range between 1.18% and 1.26% and in the mutant, the percentage of protein aggregation is 9.39% and 18.44%.

3.4. Spectroscopy Procedure

To acquire the spectra it was used MIR spectroscopy. The plasma and yeast samples were placed in the ATR crystal of FTIR spectrometer. Mid-IR absorbance spectroscopy plots the recorded intensity of absorption bands *versus* an interval of wavenumbers [150].

3.4.1. Spectral Acquisition

Previously spectroscopic analysis, the plasma samples that were stored at - 80ºC were thawed at room temperature and homogenized with vortex for approximately 8 seconds. Perkin-Elmer Spectrum BX FT-IR™ spectrometer was used to obtained spectra in the 4000-900 cm^{-1} range at 8 cm^{-1} resolution with 64 co-added scans. The room temperature and humidity during all process were maintained at ± 23ºC and ± 35%, respectively and the spectra were only obtained when these room conditions were fulfilled. The acquisition of background single beam was performed against air (empty crystal), before 8µL of plasma were placed on the ATR diamond crystal. 16 consecutive spectra were acquired, which corresponds to approximately 40 minutes being this the estimated time for drying process. Despite time consuming in this process it requires minor computational manipulations and is capable of eliminating the water spectral contribution, keeping an acceptable SNR [197]. In what concerns to yeast samples, they were withdrawn from the culture medium with a swab and placed directly in the crystal. Spectra acquisition was performed without drying. Before placed the yeast samples in the ATR diamond crystal it was acquired the background spectra against air (empty crystal).

The spectra were acquired by mid-infrared spectroscopy for each sample and between the spectral acquisitions of each sample, the diamond crystal of spectrometer must be carefully cleaned with 70% ethanol and distilled water and dried with absorbent paper. Afterward spectra acquisition, the data were submitted to multivariate analysis which allows the identification of pertinent spectra regions/bands and their corresponding assignment to chemical functional groups.

3.5. Multivariate Analysis

MIR spectroscopy provides rich spectral information that cannot be interpreted by a unique visual inspection or single peak analysis. Therefore, it is indispensable to apply appropriate multivariate data analysis techniques to support data interpretation. Multivariate data analysis

uses mathematical, statistical and computer sciences to efficiently extract useful information from data generated via chemical measurements [198]. The results of FTIR, as it was expected, provided many variables (wavenumbers) for a given a number of individuals.

Multivariate analysis methods are divided in multivariate classification (pattern recognition) and multivariate regression. Besides that, the pattern recognition techniques can be further divided in supervised or unsupervised learning procedures. The linear discriminant analysis (LDA) and artificial neural networks (ANNS) are supervised methods once *a priori* information is available regarding the data sets. Whilst in unsupervised pattern recognition, no *a priori* knowledge about the data set is required. Principal-component analysis (PCA) and cluster analysis (CA) are examples of unsupervised methods (these are exploratory methods for data analysis). On the other hand, the supervised pattern-recognition techniques allow a more precise classification within the class boundaries. Multivariate regression methods, like principal components regression (PCR) and partial least squares regression (PLS) are often applied to analyze one or multiple constituents in a complex sample that are subject to significantly overlapping analytical signals [148,151,198].

In this work, PCA and PLS were applied to the FTIR spectra. PCA is a data-reduction technique, once condense the large number of initial variables to a data set with only a few variables or principal components (PCs) reflecting the most relevant analytical information. Other characteristic of this technique is the effort in resolving overlapping spectral features [150,198]. The PCA results comprise two different plots: scores and loadings. The score plot is a projection of data onto subspace and it is used for interpreting relations between observations. The loading plot is a plot of the relationship among original variables and subspace dimensions and it is used for interpreting associations between variables [199]. PLS is a method for making predictive models when the factors are many and extremely collinear. This technique condenses the x data (variables matching to the frequency or wavelength axis of a spectrum) into less variables and at the same time includes the understanding of the y data (variables having the concentration information on multiple constituents *per* sample) along the transformation of the coordinate system into so-called factors, which are equal to PCs in PCA [198,200]. PCA and PLS were applied to the mid-IR spectra of yeast and plasma samples to specific spectral region 1800-1350 cm^{-1}, which is mostly related to protein conformation and to chemical properties of nucleic acids bases, fatty acids and carbohydrates, in order to extract the major sources of variability [197].

Chapter Three

This chapter contains the results of the application of FTIR spectroscopy to the plasma and yeast samples.

3. RESULTS AND DISCCUSSION

3.1. Yeast Samples Spectral Analysis

With the goal of studying protein aggregates and protein alterations by FTIR, a model organism it was used.

Some physiologic and pathologic mechanisms, as aging, have an universal character and they are conserved in a wide range of organisms. The main model systems used to study human mechanisms are human cells; *Saccharomyces cerevisiae*; *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus* and *Rattus norvegicus*. To study aging these organisms are required since in humans aging process it is almost impossible to follow because it is a slow process. Once these organisms have small size, short life cycles and are easy to manipulate genetically they become inexpensive subjects for studies. In this study *Saccharomyces cerevisiae* was used. It is an extensively used model of cellular and organismal aging [201–204].

The yeast samples used in this study can be divided in two different groups: wild-type and stress, in this last protein aggregation was induced.

The acquired spectra were subject of direct analysis with visualization of the spectroscopic signals related to biochemical pattern of all yeast samples. Once each spectroscopic signal is linked to a defined chemical group and/or chemical family of compound it is possible to identify and compare the main differences between the two yeast groups: wild-type and stress (figure 6).

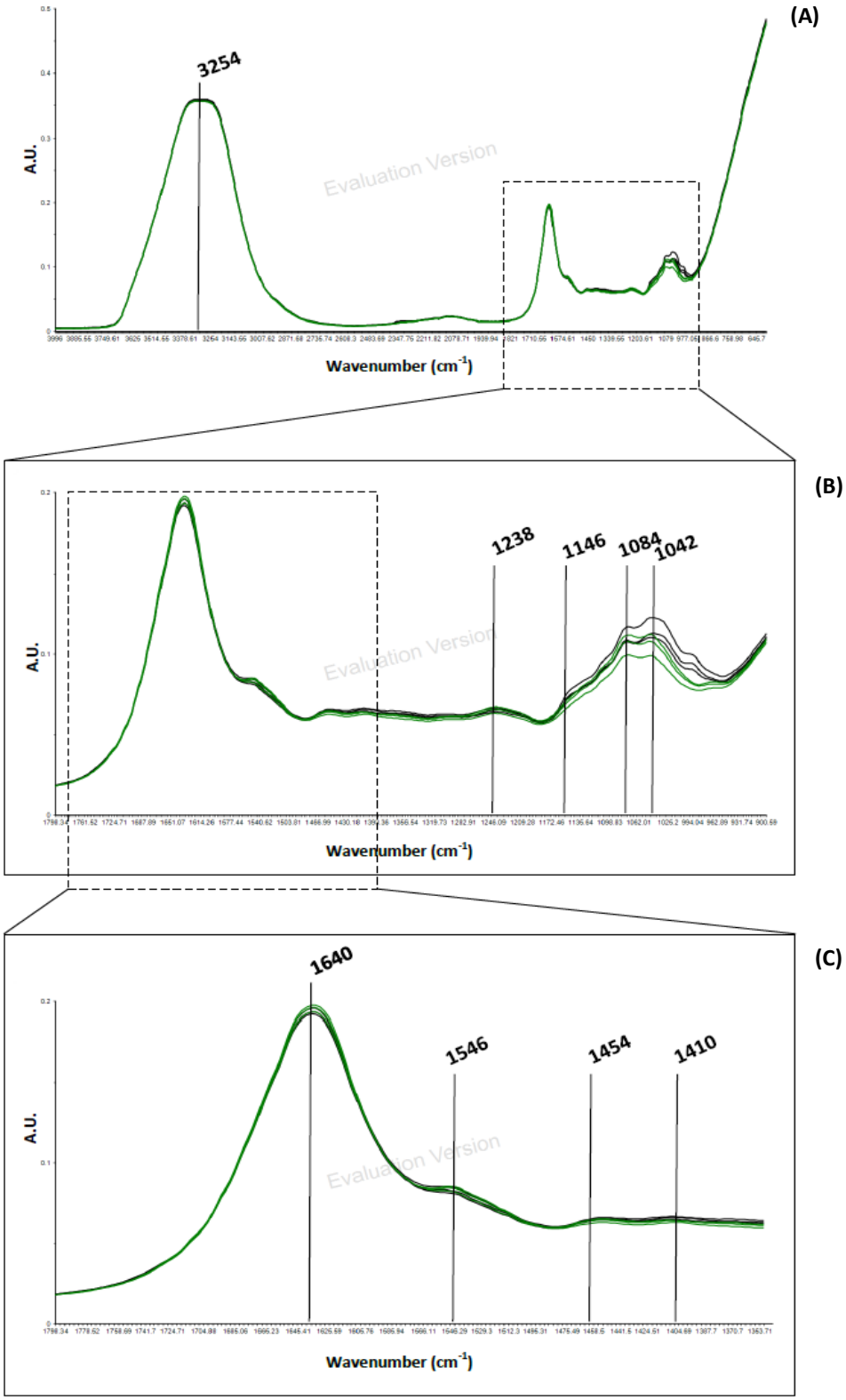

Figure 6 - The 4000-600 cm^{-1} FTIR spectral region of yeast samples divided into two different groups: wild-type (green lines) and stress (black lines) presenting the maximum wavenumber peaks. (B) Picture is a magnification of the 1800-900 cm^{-1} and (C) of the 1800-1350 cm^{-1}. X-axis: wavenumber (cm^{-1}); Y-axis: arbitrary units (A.U.)

As known, the spectral region 3500-2700 cm^{-1} is related to lipids presence (saturated or unsaturated), most fatty acids and phospholipids and also some information of amide A and B of proteins may be observed in this region. 1800-1350 cm^{-1} region is related with protein conformation mode, being this the region with more study interest and 1200-900 cm^{-1} region is assigned to nucleic acids and polysaccharides. According to figure 6 (C) the maximum wavenumber peaks identified in the 1800-1350 cm^{-1} region are 1640 cm^{-1}, 1546 cm^{-1}, 1454 cm^{-1}, 1410 cm^{-1}. In accordance to literature the assignment of some bands commonly found in microbial FTIR spectra are for 1640 cm^{-1} amide I of β-pleated sheet structures of proteins [205,206], for 1546 cm^{-1} amide II α-helix band of proteins [163,205–209], for 1454 cm^{-1} scissoring of CH_2/CH_3 [208,210] and for 1410 cm^{-1} C-O-H in-plane bending in carbohydrates, DNA/RNA backbone and proteins [205]. According to figure 6, which represents the spectroscopic profile of yeasts, there are not differences identified through the direct spectra analysis between the two groups of samples in the 1800-1350 cm^{-1} region (figure 6 (C)). The following step was to apply a PCA multivariate analysis to the 1800-1350 cm^{-1} region for all yeast samples, for a better comparison and characterization among the different sample groups. A PLS analysis is also applied to the 1800-1350 cm^{-1} region to investigate the correlation between FTIR spectra and percentage of protein aggregates. The region below 1200 cm^{-1} was not used once the induced stress in the models promotes the alteration/production of polysaccharides.

3.2. PCA Analysis and Identification of Main Spectroscopic Differences Between Sample Groups

Yeasts spectra were submitted to principal components analysis. PCA provides a score and a loading diagrams, being possible to identify and characterize the sub-groups of samples: wild-type and stress groups.

3.2.1. Spectral Range of 1800-1350 cm^{-1}

PCA was applied to the 1800-1350 cm^{-1}, specific region of spectra that allow verifying the presence of protein alteration in the samples. This spectral range is mostly correlated with protein secondary structure (amide I and amide II), even though it can be associated to chemical properties of nucleic acids, fatty acids and carbohydrates.

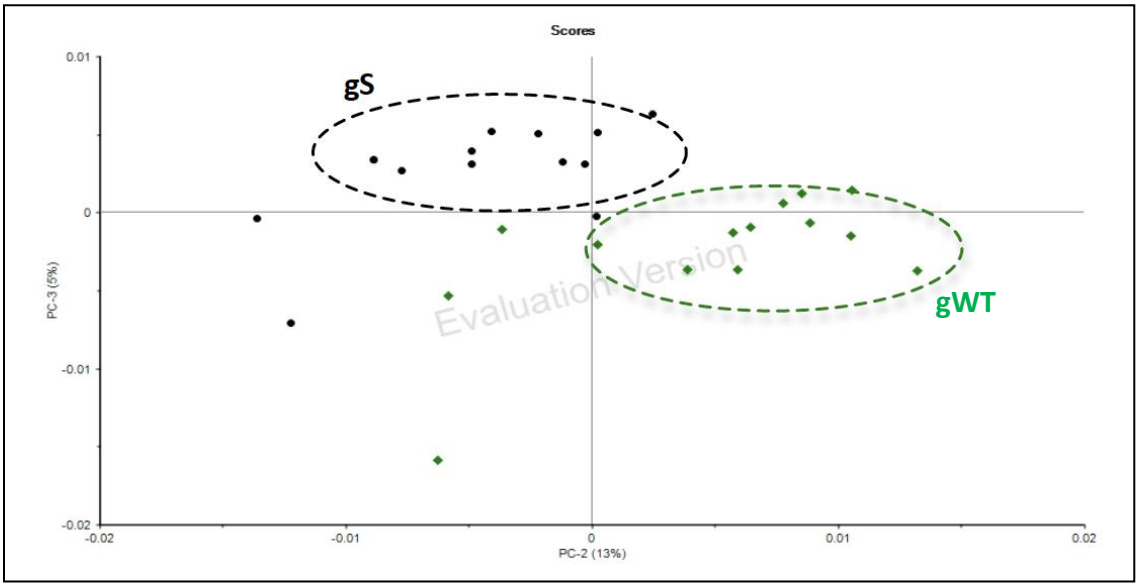

Figure 7 - PCA scores of 1800-1350 cm^{-1} spectral region showing samples distribution at a PC2 x PC3 scores diagram. In this diagram it is possible to identify the two different sample groups: wild-type (green) and stress (black). gWT: wild-type group, gS: stress group

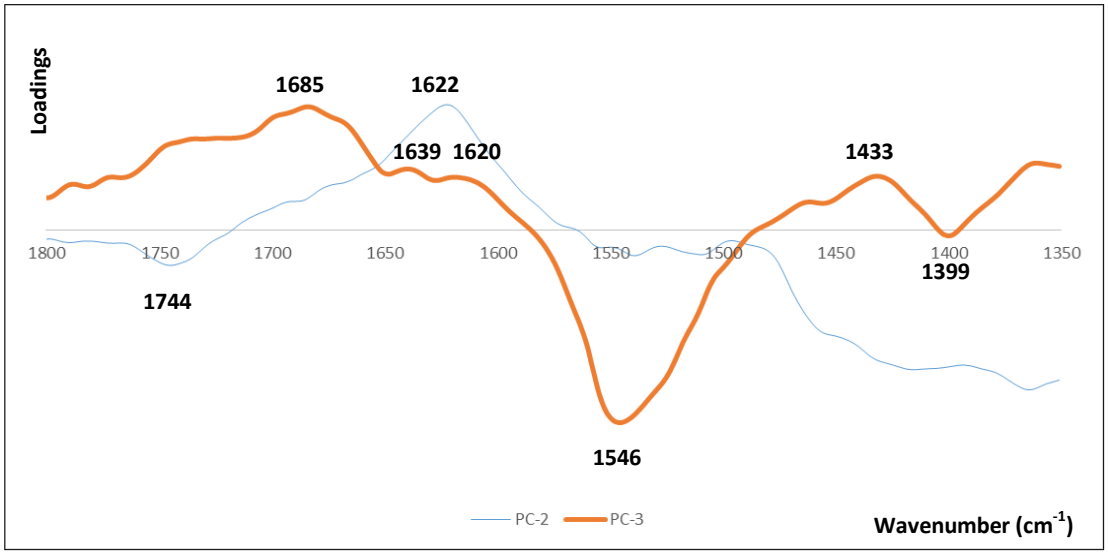

Figure 8 - PC2 and PC3 loadings profile of 1800-1350 cm^{-1} region showing the main maximum wavenumber peaks.

Through the analysis of figure 7 it is possible verify that the wild-type (gWT) samples are located mainly in negative PC3 and the stress (gS) samples are mainly in positive PC3 region. PCA loadings allow the comprehension of the distributional pattern of the samples (figure 8) and the identification of molecular alterations that might be present. For understand this discrimination it is essential the analysis of the assignments of loading signals peaks (table 3 and 4).

Table 3 - Assignments of the main maximum peaks from PC2 loadings in the 1800-1350 cm^{-1} spectral region.

Peaks (cm^{-1})	PCs	Assignment	Score (group)	References
1744	PC2 (-)	Ester C=O stretching of triglycerides, C=O stretching of polysaccharides, C=O stretching of lipids	gS	[147,211–213]
1622	PC2 (+)	Amide I band components of proteins	gWT	[163,205,209,210]

Table 4 - Assignments of the main maximum peaks from PC3 loadings in the 1800-1350 cm^{-1} spectral region.

Peaks (cm^{-1})	PCs	Assignment	Score (group)	References
1685	PC3 (+)	Amide I band components of proteins	gS	[163,205,209,210]
1639	PC3 (+)	Amide I band components of proteins	gS	[163,205,208,209]
1620	PC3 (+)	Amide I band components of proteins	gS	[163,205,208,209]
1546	PC3 (-)	Amide II band of proteins	gWT	[163,205–209]
1433	PC3 (+)	C–H bending group in fatty acids	gS	[214]
1399	PC3 (-)	C=O symmetric stretching of COO^- group in amino acids, fatty acids	gWT	[163,206–208,210]

According to figure 7 samples' main distribution occurs along PC3, being the 1685 cm^{-1}, 1639 cm^{-1}, 1620 cm^{-1}, 1546 cm^{-1}, 1433 cm^{-1} and 1399 cm^{-1} maximum wavenumber peaks responsible for the discrimination. Although, in this case, these identified peaks are only associated with a sample group, gWT or gS, this does not mean that the same peaks are not present in the other sample group.

The peaks at 1685 cm^{-1}, 1639 cm^{-1} and 1620 cm^{-1} present in stress samples are related to amide I β-sheet structures of proteins, while the 1546 cm^{-1} band, associated with amide II α-helix band of proteins, is observed in wild-type samples. The peak 1620 cm^{-1} is located in the vibrational frequency of an aggregated protein (1620-1625 cm^{-1}) [185,215]. This means that there are significant structural differences among gWT and gS in the amides regions.

As amide I band is sensitive to protein secondary structure, FTIR is commonly applied to study protein alterations, mainly protein aggregation and misfolding *in vitro*, which has been widely used to monitor amyloid fibril formation in *Escherichia coli* samples. FTIR spectroscopy is a well-established technique, which has been shown to be sensitive to the secondary structure of proteins, becoming a valuable technique to assess the presence of protein aggregates *in vitro*. Due to unique hydrogen bonding environments for the diverse secondary structure elements, shifts are found in the frequency of the amide I band [185,216–218].

1433 cm^{-1} band is found in stress samples and is related with C–H bending in fatty acids and the 1399 cm^{-1} band is associated with C=O symmetric stretching of COO^- group in amino acids and fatty acids and is present in wild-type samples. This suggests that although the two groups of

samples contained fatty acids in their composition they have present differences that FTIR was able to detect.

These preliminary results confirming that protein conformational changes can be detected by FTIR and this spectroscopic technique may be used in follow up protein changes related to aging process.

The PCA analysis allowed verifying that there are differences in proteins spectral region (1800-1350 cm^{-1}) between the wild-type and stress samples. So, in the following step it was applied a PLS analysis to all spectroscopic data (represented in figure 6) in the 1800-1350 cm^{-1} spectral region with the goal to study the correlation between FTIR spectra and percentage of protein aggregates.

3.3. PLS Analysis to Evaluate the Correlation Between FTIR and Percentage of Protein Aggregates Evaluated by Microscopy

PLS analysis was applied to 1800-1350 cm^{-1} spectral region in order to predict the correlation between FTIR spectra and percentage of protein aggregates in yeast samples (figure 9 (A)). A correlation with a determination coefficient of R^2=0,65 was observed with a calibration of 4,06% RMSEC and a prevision error of 4,52% RMSEP, the model was built with two latent variables, meaning that the biggest part of the variation of Y is linearly explained by X (model appropriate).

This PLS model gives the information about the spectral signals that are correlated to the percentage of protein aggregates (figure 9 (B)) that are identified in table 5. The positive peaks identified in coefficient B are related to the stress samples, the ones with higher percentage of protein aggregates.

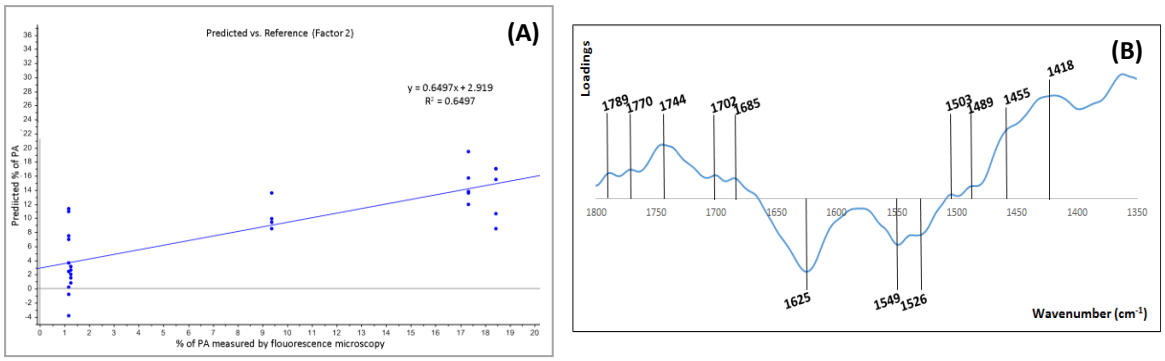

Figure 9 - (A) Correlation between percentages of real values of protein aggregates and predictive ones. (B) Coefficient B was obtained with two latent variables.

Table 5 - Assignments of the identified maximum wavenumber peaks from PLS (1800-1350 cm^{-1}).

Peaks (cm^{-1})	Coef. B	Assignment	References
1789	**+**	C=O stretching affected by Cl, etc. (not clear which are biomolecule contributors)	[205,207]
1770	**+**	C=O stretching affected by Cl, etc. (not clear which are biomolecule contributors)	[205,207]
1744	**+**	Ester C=O stretching of triglycerides, C=O stretching of polysaccharides, C=O stretching of lipids	[147,211–213]
1702	**+**	C=O stretching, H-bonded in DNA, RNA	[147,205,219]
1685	**+**	Amide I band components of proteins	[163,205,208,209]
1625	**-**	Amide I band components of proteins	[163,205,208,209]
1549	**-**	Amide II band of proteins	[163,205–209]
1526	**-**	Amide II band of proteins	[163,205–209]
1503	**+**	$>CH_2$ and $>CH_3$ bending modes of lipids and proteins	[178]
1489	**+**	C-H deformation*	[147,220]
1455	**+**	C-H deformation of >CH2 in lipids, proteins	[205,207]
1418	**+**	C-O-H in-plane bending in carbohydrates, DNA/RNA backbone, proteins	[205]

*Characteristic band assignments of biological samples.

Proteins in a non-native conformation could engage in aberrant interactions with other cellular components and/or aggregate. The misfolded proteins are cytotoxic, so several neurodegenerative and age-related disorders are related with misfolding and aggregation. The majority of the knowledge on protein aggregation arises from *in vitro* studies. There are some studies on aggregation in living cells. For example, it was already identified around 200 aggregated proteins in stationary phase *Saccharomyces cerevisiae* [221–223]. Nevertheless, aggregation is not necessarily a dead-end situation for a protein *in vivo*. It has been observed in cells from different species disaggregation followed by refolding of aggregated proteins [223].

According to figure 9 (B) spectroscopic signals correlated with protein alterations and protein aggregation were identified in stress and wild-type samples. The main bands identified in PLS are 1685 cm^{-1}, 1625 cm^{-1}, 1549 cm^{-1} and 1526 cm^{-1}. The peak at 1685 cm^{-1} is related with amide I β-sheet structures and represents stress samples. The vibrational frequency of an aggregated protein falls around 1620–1625 cm^{-1} [185,215]. In humans it was already verified through FTIR that amyloid plaques have elevated β-sheet content, as demonstrated by a strong amide I absorbance at 1625 cm^{-1} in brain tissue samples [215].

The 1625 cm^{-1} band, in human brain tissues, is considered a shift in amide I bands due to protein aggregates [215]. However, in this study, it is related with wild-type samples with less

amount of protein aggregates, being this an opposite result of what was expected to have, once it was expectable that 1625 cm^{-1} band was related with stress samples, samples with high amount of protein aggregation. The 1549 cm^{-1} and 1526 cm^{-1} bands are related with amide II α-helix structures in wild-type samples. So, it is possible claim that the principal differences are in the amide I (1700-1600 cm^{-1}) and amide II (1550-1450 cm^{-1}) regions reflecting protein conformational changes. Nonetheless, it is necessary to perform additional studies to understand better the relationship between the presence of protein aggregates in the samples and the presence of FTIR spectroscopic signals, for example by characterization of protein fraction by SDS-PAGE.

FTIR spectroscopy has shown to be capable of discriminating between wild-type and stress yeast samples in the protein region, more concretely in amide I region, reflecting protein conformational changes.

Consequently, FTIR was applied to human plasma samples of volunteers with age between 65 and 82 years old with the main goal of identify and characterize protein alterations.

3.4. Spectral Analysis of Human Plasma Samples

The proteins present in the human plasma are a mixture of complement products, lipoproteins and other conjugated proteins called “plasma proteins”. The three major fractions of plasma proteins are albumin, globulins (α1-globulin, α2-globulin, β-globulin and ¥-globulin) and fibrinogen. Serum albumin is the most abundant protein in the mammal’s serum and has a role as a molecular chaperone being able to prevent misfolding and/or protein aggregation [224,225].

Each one of spectral signals have one or more assignments (specific type of molecular vibration), that match to a defined chemical group and/or chemical family compound. This correspondence allows the identification of principal chemical components of whole plasma samples (table 6). After acquisition, spectra of plasma samples were subject to direct analysis with visualization of main spectroscopic signals related to chemical pattern of all plasma samples. Figure 10 presents all spectra of elderly plasma samples used in this study.

The mid-infrared region can be divided in smaller spectral regions of interest where strong absorption bands are related with specific components. The regions include: fatty acids region; amide region; (primarily to proteins and peptides); mixed region; (ascribed to carboxylic groups of proteins); free amino acids and polysaccharides and polysaccharides region. In addition, there are other spectral regions like the one which is relevant to RNA, DNA and phospholipid content [200]. In the 900-600 cm^{-1} region some bands arise from aromatic ring vibrations of phenylalanine, tyrosine, tryptophan, and various nucleotides [165]. In order to look in detail to the spectroscopic

differences associated with age, spectra of plasma of 65 years old donor (newest of the data set) was compared with one of 82 years old (oldest of the data set). The identification of major differences was identified in figure 11.

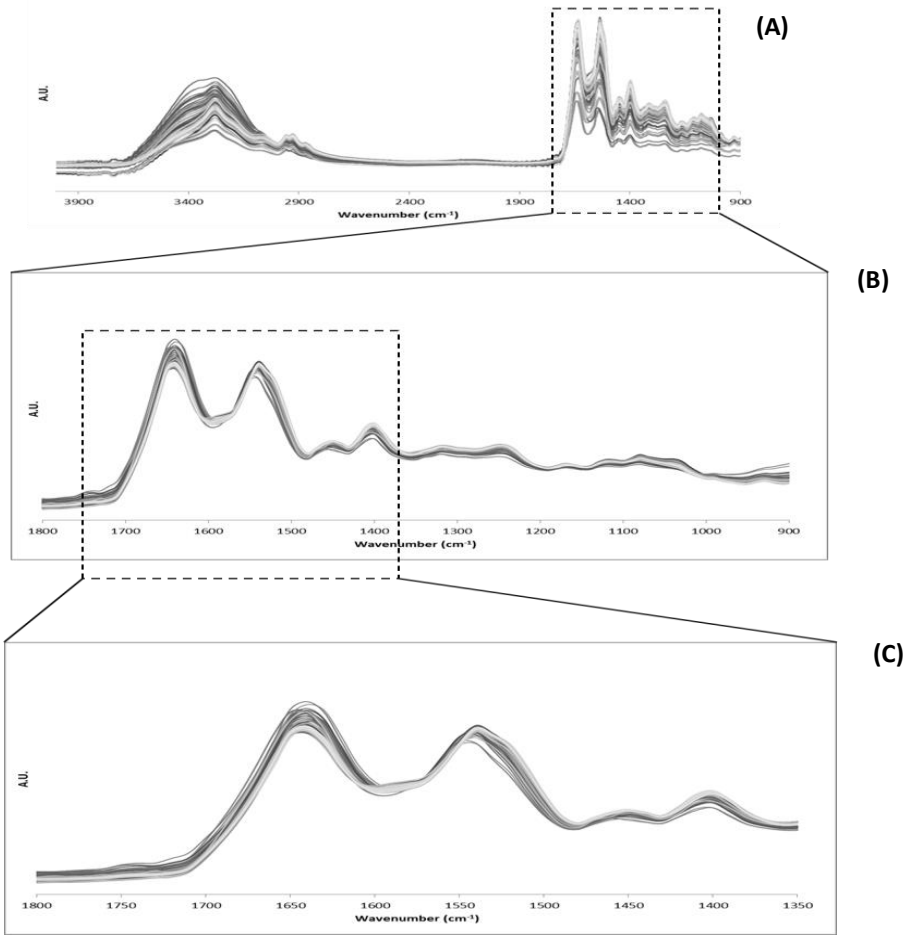

Figure 10 - FTIR spectra of all plasma samples used in this study, in a wavenumber of 4000-900 cm^{-1} (A). The FTIR spectral regions of 1800-900 cm^{-1} (B) and 1800-1350 cm^{-1} (C) were magnified once they had study interest. This figure represents the 60 spectra that include the 6 replicates of the 10 samples that comprise the data set. X-axis: wavenumber (cm^{-1}); Y-axis: arbitrary units (A.U.)

Table 6 - Spectral assignments of plasma samples.

Peaks (cm^{-1})	Assignments	Plasma Contents	References
≈ 3454	N-H stretching	Proteins (amide A)	[226]
≈ 3286	N-H stretching, H-O-H symmetric and asymmetric stretching*	Proteins (amide A)	[227,228]
≈ 3072	C-H stretching	Aromatic ring	[147,229]
≈ 2962	C-H asymmetric stretching of CH_3 group*	Lipid acyl chains in lipid bilayers (phospholipids)	[163,227,230,231]
≈ 2938	C-H stretching of CH_2 and CH_3 group*	Fatty acids	[163,226,227]
≈ 2872	C-H symmetric stretching of CH_3 group	Lipids (long chain fatty acids, phospholipids)	[191,232,233]
≈ 1740	C=O stretching	Fatty acids esters (triglycerides), lipid bilayers (phospholipids)	[137,227,234]

≈ 1716	C=O stretching	Carbonic acid, phospholipid sub-structures, purine bases from nucleic acids	[163,227,234,235]
≈ 1694	C=O stretching in-plane coupled to the C-N stretching and N-H bond in-plane bending	Proteins (amide I)	[147,236]
≈ 1680	Unordered random coils and turns of amide I	Proteins (amide I)	[147,236]
≈ 1644	-	Proteins (amide I)	[147,236,237]
≈ 1636	C=O stretching and N-H in-plane bending	Proteins (amide I)	[238,239]
≈ 1570	-	Proteins (amide II)	[147,236,240]
≈ 1550	C-N stretching and N-H bending	Proteins (amide II)	[147,241,242]
≈ 1536	N-H in-plane bending, C-H stretching*	Proteins (amide II)	[163,227,228]
≈ 1516	N-H in-plane bending and C-H stretching	Proteins (amide II)	[238,239]
≈ 1512	CC (phenyl ring) stretching; CH in-plane bending*	Tyrosine band (proteins side chain)	[163,227,243]
≈ 1470	CH_2 bending; C-H asymmetric bending; CH_2 symmetric in-plane bending (scissoring)*	Proteins; Carbohydrates; Lipid acyl chains in lipid bilayers (phospholipids)	[147,163,231,234]
≈ 1452	CH_2 bending and CH_3 asymmetric in-plane bending (rocking)*; C-O symmetric stretching	Proteins, membrane lipids (phospholipids); Carbohydrates	[147,230,231,234]
≈ 1398	C=O symmetric stretching of COO^- group; C-O symmetric stretching*; C-O bending (carboxylate ions)*; CH_3 and $N^+(CH_3)_3$ symmetric bending*	Proteins and carbohydrates; Mixed region (carboxylic groups); Membrane lipids (phospholipids)	[228,230,231,234]
≈ 1396	CH_3 symmetric bending of the CH_3 groups of proteins	Proteins	[147,244]
≈ 1310	-	Proteins (amide III)	[147,245]
≈ 1242	N-H in-plane bending, C-N stretching*; P=O asymmetric stretching of PO_2^- group	Proteins (amide III, mainly α-helix conformation); Phosphodiester groups of nuclei acids	[147,227,228]
≈ 1168	Ester and CO-O-C antisymmetric stretching*; C-O, C-C stretching and C-O-H, C-O-C deformation*	Membrane lipids (phospholipids); Carbohydrates	[227,228,231]
≈ 1115	C-O, C-C stretching, C-O-H, C-O-C deformation and C-O-C asymmetric stretching*; P-O-C symmetric stretching, C-O stretching of C-OH group	Carbohydrates; Phosphodiesters; RNA	[147,227,234]
≈ 1077	C-O, C-C stretching, C-O-H, C-O-C deformation and C-O-C asymmetric stretching*; Po_2^-, CO-O-C symmetric stretching*	Carbohydrates; Membrane lipids (phospholipids) and nucleic acids sugar backbone	[147,227,231,234]
≈ 1032	C-O, C-C stretching, C-O-H, C-O-C deformation*; C-O symmetric stretching	Carbohydrates; Nucleic acids sugar backbone	[147,227,234]
≈ 987	C-O; C-C stretching; C-H-O, C-O-C deformation, C-H bending*; CH_3-N asymmetric stretching	Carbohydrates; Membrane lipids (phospholipids	[227,234]
≈ 924	C-O; C-C stretching; C-H-O, C-O-C deformation*; $N^+(CH_3)_3$ symmetric stretching*	Carbohydrates; Membrane lipids (phospholipids)	[227,231]

*Characteristic band assignments of biological samples (not specific for plasma).

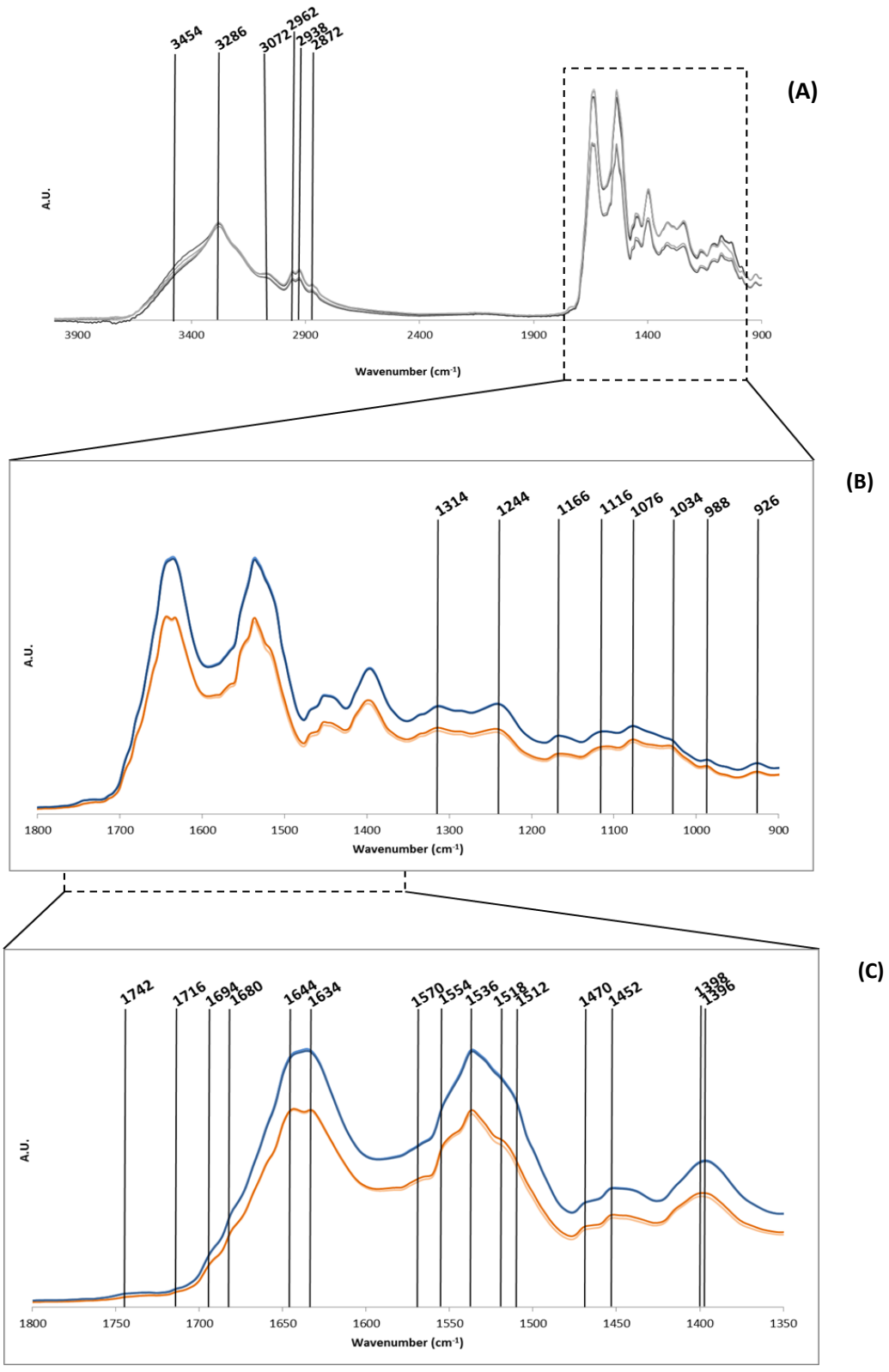

Figure 11 - 4000-900 cm^{-1} FTIR spectral region of two plasma samples, one of a 65 years donor (blue line) and other of a 82 years donor (orange line), presenting maximum wavenumber peaks. (B) Picture is a magnification of the 1800-900 cm^{-1} and (C) of the 1800-1350 cm^{-1}. X-axis: wavenumber (cm^{-1}); Y-axis: arbitrary units (A.U.)

According to signals observed in spectra (figure 10 and 11) table 6 was created. It provides a bibliographic review of the assignments that have been published until now in the infrared spectroscopy field about assignments of the mid-IR spectrum associated to biological samples. The mid-IR spectroscopy is a great tool for study biological molecules and through this technique it is possible identify the following main groups of these molecules: proteins and peptides, lipids, carbohydrates and nucleic acids [148,153,184].

Infrared spectra of proteins exhibit absorption bands related to their characteristic amide groups. There are nine amide bands, amide A, amide B and amides I-VII, however, only amides A, I, II and III were identified in this study. Amide A and amide B appear at approximately 3300 cm^{-1} and 3100 cm^{-1}, respectively and are due to N-H stretching vibration. Amides I and II are the main bands in the protein infrared spectrum, being amide I the most important to study secondary structure. Amide I IR bands are located in the region 1700-1600 cm^{-1}. This band represents 70–85% of C=O stretching vibration of the amide group coupled to in-phase bending of the N–H bond and stretching of the C–N bond. Amide II band region shifts from 1550-1450 cm^{-1} and represents 40-60% of the N-H bending and 18-40% of the C-N stretching vibrations. Amide III can be located at 1250-1300 cm^{-1} region and its band is the combination of C-N stretching, N–H bending, C=O stretching and O=C–N bending. The amides III and IV are very complex bands resulting from mixtures of several coordinate displacements. In amides V-VII it is possible to see out-of-plane motions. In mid-IR analysis amide IV-VII bands are not of importance and are bands with very low intensities [148,153,228].

Lipids are important molecular components of membranes and have characteristic C-H stretching vibrations located among 3025-2700 cm^{-1} region in FTIR spectrum. IR bands due to the terminal CH_3 functional groups appear at 2957 cm^{-1} (asymmetric stretching) and 2872 cm^{-1} (symmetric stretching). Triglycerides, phospholipids and cholesteryl esters, present in blood serum, are the classes of lipids identified in mid-IR analysis. These classes of compounds can be characterized by their carbonyl bands: at 1742 cm^{-1} for the triglycerides, at 1737 cm^{-1} for the phospholipids and at 1723 cm^{-1} for the cholesteryl esters representing the peak maxima [148,235].

IR bands associated to carbohydrates are related to hydroxyl and carbonyl groups and acetal bonds. The carbonyl group bands can be identified at the 1650-1850 cm^{-1} region which corresponds to the C=O stretching vibration. In the 950-750 cm^{-1} region of mid-IR spectrum it is possible to distinguish the correspondent bands for α and β conformers or pyranoid and furanoid ring vibrations of monosaccharides and polysaccharides [153,242].

FTIR spectra give as well information about nucleic acid biochemistry. DNA and RNA bases represent the purine and pyrimidine vibrations in 1800-1500 cm^{-1} region. In addition, the following characteristic findings are representative of a FTIR spectra of nucleic acids: C=O stretching vibrations from purine and pyrimidine at the wavenumbers 1717 cm^{-1} and 1666 cm^{-1} respectively, and also antisymmetric and symmetric PO_2^- stretching vibrations at the region 1500-1000 cm^{-1} [148,235]. The detailed description of the spectra, summarized in table 6, helps to interpret figure 10 and to explain the differences detected in figure 11. These figures present spectra in the 4000-900 cm^{-1} region.

In this precise region (figure 10 (C)) one of the main peaks is around 1740 cm^{-1}, which is related with C=O stretching in lipids and ester C=O stretching in phospholipids [147,246]. Peak at 1640 cm^{-1} is related with amide I β-sheet band of proteins [147,241], 1540 cm^{-1} is associated with amide II absorption [147,236], 1450 cm^{-1} is related with asymmetric CH_3 bending modes of the methyl groups of proteins [147,247] and 1400 cm^{-1} is associated with symmetric stretching vibration of COO^- group of fatty acids and amino acids and symmetric stretching of CH_3 groups in proteins [147,248,249]. In order to have a better comparison between samples, making evident the most significant differences upon age, spectra of two plasma samples were selected. The chosen samples represent the 65 years sample which corresponds to the youngest donor of the data set and the 82 years sample corresponds to the oldest donor of the data set. These spectra allowed to identify the main protein differences in plasma related to age (figure 11).

According to figure 11, which represents the spectroscopic profile of plasma of the 65 and 82 years volunteers, the most significant identified differences are in the regions 1644-1634 cm^{-1} and 1570-1518 cm^{-1}. Protein spectra have two major features, amide I (1700-1600 cm^{-1}) and amide II (1550-1450 cm^{-1}) bands. Amide I bands assume increase sensitivity to the alteration of protein secondary structure.

The wavenumbers 1644 cm^{-1} and 1634 cm^{-1} are associated to amide I and the wavenumbers 1570 cm^{-1}, 1554 cm^{-1}, 1536 cm^{-1} and 1518 cm^{-1} with amide II. These main differences might be due to conformational changes in proteins that involve secondary structure of amides. Amide I β-sheet structures have vibrational frequencies approximately at 1610–1640 cm^{-1} and/or 1680–1700 cm^{-1} and random coil structures around 1640–1650 cm^{-1} [148,250]. So it is possible to assume that 1644 cm^{-1} represents a random coil and 1634 cm^{-1} peak a β-sheet [148,250]. In amide II random coil, α-helix and turn structures fall around 1504–1525 cm^{-1}, 1527–1560 cm^{-1} and 1564–1583 cm^{-1}, respectively [250]. Therefore, the wavenumber 1570 cm^{-1} represents a turn, 1554 cm^{-1} and 1536 cm^{-1} a α-helix and 1518 cm^{-1} a random coil [250]. It is Known that β-sheet conformation

is more prone to form aggregates [251], so it is possible to conclude that the 1634 cm^{-1} could be related with aggregates.

After direct spectral analysis, with the purpose to investigate the correlation between age and protein alterations/modifications it was applied a PLS analysis to all spectra of the data set (represented in figure 10) in the 1800-1350 cm^{-1} spectral region, assigned mainly to proteins amide I and II.

3.5. PLS Analysis Allows the Correlation Between Age and Protein Alterations

PLS was applied to 1800-1350 cm^{-1} spectral region to evaluate the correlation between age and proteins spectral region of human plasma samples (figure 12 (A)). A correlation with a determination coefficient of R^2=0,68 was observed with a calibration of 10,88% RMSEC and a prevision error 6,72% RMSEP. The model was built with two latent variables indicating that the majority of the variation of Y is linearly explained by X (model appropriate). This PLS provides information about the spectral signals that are related to age (figure 12 (B)), which are identified and characterized according to its assignments in table 7. The positive peaks of coefficient B are related with samples from older volunteers and the negative peaks with younger volunteers.

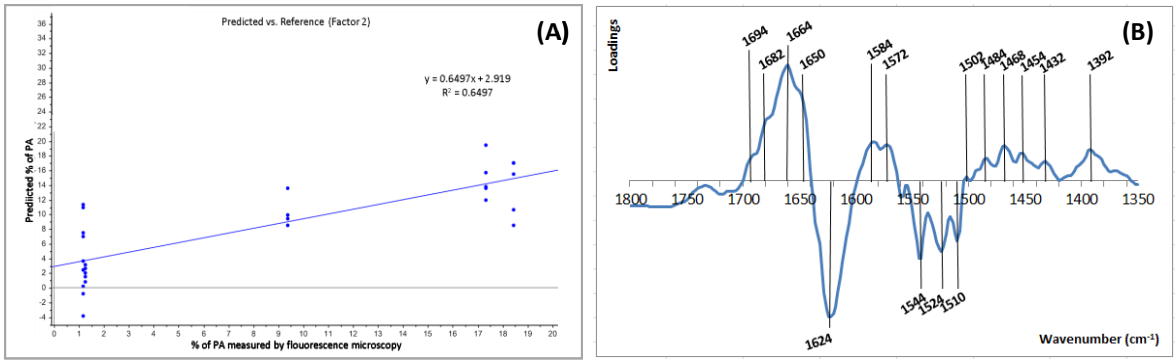

Figure 12 - (A) Correlation between real values of age and predictive ones. (B) Coefficient B was obtained with two latent variables.

Table 7 - Assignments of the major maximum wavenumber peaks from PLS (1800-1350 cm^{-1}).

Peaks (cm^{-1})	Coef. B	Assignments	References
1694	**+**	C=O in-plane stretching coupled to the C-N stretching and N-H bond in-plane bending (antiparallel β-sheet amide I)	[147,236]
1682	**+**	C=O guanine deformation N-H in-plane; Unordered random coils, turns and loops (amide I)	[147,226,252]
1664	**+**	C=O band of α-helical structure (amide I)*, turns and loops	[226,242]
1650	**+**	C=O symmetric stretching band of α-helical structure (amide I)	[147,227,233]
1624	**-**	C=O stretching band of parallel β-sheet (amide I)	[226,235,252]

1584	**+**	C=N imidazole ring stretching (nucleic acids); Benzene ring vibrations from arginine	[148]
1572	**+**	NH_3^+ symmetric bending (membrane lipids); C=N imidazole ring stretching (nucleic acids)	[148,230,231]
1544	**-**	N-H in-plane bending and C-N stretching (amide II)	[147,228,233]
1524	**-**	N-H in-plane bending and C-N stretching (amide II); Stretching C=N, C=C	[147,219,228]
1510	**-**	N-H in-plane bending and C-N stretching (amide II); C-H in-plane bending of phenyl rings (tyrosine)	[226,243,253]
1502	**+**	C-H in-plane bending; C=C stretching; o-Tyr	[147,254]
1484	**+**	$N^+(CH_3)_3$ antisymmetric bending (lipids); NH_3^+ bending from lysine	[153,231]
1468	**+**	CH_2 scissoring and CH_3 scissoring and asymmetric bending of phospholipids (membrane lipids); CH_2 bending (lipids, proteins); CH_2 in-plane bending (lipids)	[147,148,227]
1454	**+**	Asymmetric CH_3 deformation	[147,248]
1432	**+**	CH_2 in-plane bending (polysaccharides); C–O–H in-plane bending (carboxylic acids)	[147,148]
1392	**+**	C=O stretching of COO^-; CH_3 symmetric bending (nucleic acids)	[228,244]

*Related to structural analysis of synthetic polypeptides.

Amide I (1700-1600 cm^{-1}) and amide II (1550-1450 cm^{-1}) bands are the two most prominent vibrational bands of the protein backbone [238]. The amide I is due to C=O stretching vibration and amide II to N-H bending and C-N stretching vibrations. Amide I bands concentrated among 1650 cm^{-1} and 1660 cm^{-1} are usually considered to be characteristic of α-helix structures, bands between approximately at 1610-1640 cm^{-1} and 1680-1700 cm^{-1} are characteristic of β-sheet structures and bands at 1660 cm^{-1} and 1680 cm^{-1} are characteristic of turn structures [250,255]. 1694 cm^{-1} and 1624 cm^{-1} spectroscopic signals identified in figure 12 (B) are related to β-sheet forms of amide I, the peak 1664 cm^{-1} are related with a turn and 1650 cm^{-1} with α-helix. The peak at 1682 cm^{-1} is related with unordered random coils, turns and loops of amide I. The vibrational frequency of an aggregated protein falls around 1620-1625 cm^{-1} [185,215]. The 1624-1630 cm^{-1} region in figure 12 (B) belongs to the typical amide I β-structure band of the amorphous aggregates [252]. So it is visible through the analysis of table 7 and figure 12 (B) that there are many protein conformational differences in the amide I spectral region. It is known that aging is characterized by the decline of proteostasis leading to changes in proteins.

The bands 1584 cm^{-1} and 1572 cm^{-1}, correlated to the oldest, are probably related to alteration in conformational state and structural stability of nucleic acids molecules. In addiction 1572 cm^{-1} band is also associated to structural changes in lipids [148,231]. Many data support that

the amyloid proteins exhibit a high binding affinity for nucleic acids. Nucleic acids have been studied to be capable of inducing and accelerating significantly aggregation of amyloid proteins and the binding between nucleic acids and amyloid proteins leads to several variations in conformation of these proteins [256].

The 1544 cm^{-1} is related with amide II α-helix and 1524 cm^{-1} and 1510 cm^{-1} with amide II random coil. These bands are associated with unaltered protein conformation of amide II [226,233,250]. The 1510 cm^{-1} band is also related with free tyrosine which is a common precursor of biological amines [230].

1484 cm^{-1} and 1468 cm^{-1} bands positively correlated with the samples of the oldest volunteers are related to structural properties of lipids, mainly with membrane lipids such as fatty acids and phospholipids being these the principal targets of free radicals, oligomers and other damage-inducing agents [147,227,228,231]. Amyloid deposits from many human diseases contain membrane lipids. In most amyloid diseases, protein aggregation has been related with membrane disruption in cells and in model lipid systems. It has been observed that protein adsorption to lipid membranes, and that the lipid membranes intervene with aggregation process. Besides that, protein in diverse aggregation states (monomeric, oligomeric or fibrillary) may cause changes in membrane permeability and morphology. Furthermore, there is great evidence that lipids have a crucial role in the pathology of Parkinson's disease [257].

According to figure 12 (B), the 1624 cm^{-1} band, which represents a β-sheet structure of amide I, is located in the vibrational frequency of protein aggregates (1620-1625 cm^{-1}) and is related to the newest volunteers. Although, the obtained results are not what were expected since, our assumption was that protein aggregation increases with age, we cannot rule out the hypothesis that this spectroscopic signal shows a shift in the spectra reflecting conformational changes in the 1620-1625 cm^{-1} protein aggregation spectral region. Thus, it is possible to affirm that there is a correlation between age and the protein spectrum, suggesting that there are conformational changes with age however these need to be studied with greater detail. Relatively to the remaining results it is important to emphasize that nucleic acids could be one of the major inductors of aggregates and lipids have a crucial role in neurodegenerative diseases associated with protein aggregates.

As organisms age, the balance of gene expression levels, quality control, and protein disposal is disturbed. So, aging process leads to an age-related impairment of proteasome structure and function, due to protein damages occurring over the aging. Regulation of proteostasis has a crucial role in preventing protein aggregation which might be due to a decrease of efficacy with

age in the cellular systems responsible for protein degradation, to changes in chaperones levels in older and aging is likewise related with higher levels of oxidative stress, leading to irreversible oxidation of proteins, which impairs their degradation. These age-dependent alterations in protein homeostasis can be responsible to the aberrant aggregation of proteins in neurodegeneration and amyloidosis contexts. Aberrant protein aggregation is considered the hallmark of a wide range of age-related diseases such as Alzheimer's Parkinson's and Huntington's diseases, yet it is still known whether proteins aggregate with age in a non-disease context [185,251,258]. Diminished protein solubility and a predisposition to aggregate are observed during physiological aging but the causes are currently unknown [259].

Protein aggregation is the abnormal combination of misfolded proteins into larger, frequently insoluble structures. Aggregation is classified in two different categories: amyloid and amorphous. The amorphous protein aggregation is characterized by unordered aggregation of proteins, with each individual protein not commonly related with disease when aggregated. Amyloid protein aggregation is an extremely structured, insoluble, fibrillary deposit, generally consisting of several repeats of the same protein. To safeguard proteostasis, cell have molecular chaperones that are capable of detecting non-native misfolded proteins and act upon them to avoid amyloid formation and aggregation [260]. FTIR spectroscopy is sensitive to the secondary structure of proteins, so it is a potential technique for studying protein aggregation [185]. Many studies have been performed to prove that protein aggregates increase with age and indeed represent one aging factor [261,262].

4. CONCLUDING REMARKS

The interests to study the relationship between aging and protein aggregates and assess the capacity of FTIR in identify protein aggregates are growing, once ever more humans want to extend and improve lifespan. Most neurodegenerative diseases and other age-related diseases are largely associated with protein aggregates, so their study may lead to improvement in disease progression. FTIR is a rapid and non-destructive easy to use tool that presents low associated costs and it is widely used in studies related with aging and protein aggregates.

Plasma samples from volunteers with age between 65 and 82 years, with the goal to identify main protein conformation differences, through FTIR, were used in this work and were also studied in previous works of the research group [184,197,263,264]. Yeast samples, wild-type and stress (induced protein aggregation) groups, were also used. Model organisms are extremely

important in physiologic and pathologic studies once it is possible in these follow all aging process.

In this work, it was possible to evaluate the ability of FTIR to identify protein aggregates/alteration in protein conformation in yeasts, providing that this spectroscopic technique combined with adequate multivariate analysis tools, can be a useful screening method for posterior metabolomics/proteomic studies interested in follow protein aggregation with aging.

Direct analysis of human plasma spectra allowed identifying the principal chemical FTIR assignments in plasma, which is crucial to creating a pattern of chemical vibrations and recognizing the principal absorption peaks in the 1800-1350 cm^{-1} region that could give significant information. Since biological samples have great complexity, direct analysis is not sufficient to characterize and identify the different samples used in this study and besides that it is also not capable of identifying proteins changes and to correlate these with age. So, multivariate data analysis techniques were applied to draw out the significant and no redundant information.

IR spectra can be divided in several regions with important information, for example, the 3500-2700 cm^{-1} region represents especially lipids (saturated or unsaturated) particularly fatty acids and phospholipids, the 1800-1350 cm^{-1} region is related with protein conformation allowing identifying the protein structures and protein aggregates and the 1200-900 cm^{-1} region is associated to carbohydrates and nucleic acids and also some spectra signals present in this region could be associated with membrane lipids. In this work 1800-1350 cm^{-1} spectral region was studied. In this region, where protein signals can be observed, was found the presence of protein aggregates and these have preferentially β-sheet structures. Relatively to plasma samples it is important consider that the presence of clinical factors could have some impact in the results, for example the use of medication capable of disturbing biochemical plasma levels or unknown genetic predisposed factors.

This thesis allowed conclude that FTIR is capable of identifying different changes in the proteins spectral region, these structural differences are located predominantly in the amides region; mostly in amide I region and the β-sheet structures have greater tendency to be related with protein aggregates. It is known that aging leads to loss of proteostasis, so it can be also concluded that there is a positive correlation between age and protein alterations.

As was mentioned above, FTIR spectroscopy analysis has showed to be able in identify the protein differences and protein aggregates in different samples. Additional studies and complete clinical trials will be required to prove effectively that aging and protein aggregates are directly

related and posteriorly it will be possible to contribute with knowledge to design medicines and treatments anti-aging.

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